JOURNAL OF THE NATIONAL CANCER INSTITUTE

NATIONAL INSTITUTES OF HEALTH • Public Health Service

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Volume 21
Number 3

September 1958

Glenn Horner Algire

1907–1958

Laboratory of Biology

National Cancer Institute

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THE DEATH of Glenn Horner Algire April 28, 1958, after a protracted illness, left his many close associates and friends with a feeling of profound loss. During the 17 years he had been at the National Cancer Institute he had established an international reputation for his development of meticulous and elegant transparent-chamber and diffusion-chamber methods. These methods allowed tissue and cell behavior and reactions to be observed and studied in transparent chambers inserted under the skins of the living mice. The interruption of his work by his death is a great loss in the advancement of our knowledge of cell and tissue reactions within the living animal.

Glenn was born November 5, 1907, in Baltimore, Maryland, the son of Charles T. and Helen Newman Algire. His early childhood and grammar school days were spent with his parents, an older brother, and a younger sister. After his family moved to Blue Ridge Summit, Pennsylvania, Glenn attended and graduated from Waynesboro High School, Franklin County, Pennsylvania. With the expectation of making teaching his profession, Glenn attended Maryland State Teachers College at Towson, Maryland, from which he received a teaching certificate. After teaching and serving as principal for two years in a rural Maryland school, he attended Teachers College, Columbia University, from which he received his B.S. in 1930. During this time Glenn's interests were turning definitely toward chemistry, physics, and biology. He obtained a position as teacher of chemistry and physics at Northport High School, Long Island, where he worked during the winter sessions from 1930 to 1935. Meanwhile, during the summers he took courses, served as Research Assistant in Clinical Bacteriology at The Johns Hopkins School of Medicine, assisted in the organization of the Laboratory of Clinical Bacteriology at Duke University, and did part-time voluntary work in Clinical Pathology, United States Veterans Administration Hospital, Northport, Long Island.

With the increasing definite formation of his interests, Glenn entered the University of Maryland Medical School in 1935, completing his M.D. in 1940. Meanwhile, he was a Weaver Fellow in Gross Anatomy, working particularly under the supervision of Dr. Eduard Uhlenhuth, Head of the Department of Anatomy, and each summer at the Marine Biological Laboratory, Woods Hole, Massachusetts, Glenn worked under Dr. Uhlenhuth's direction. He continued as a Weaver Fellow all through his internship.

It was in his work with Dr. Uhlenhuth on the cells and circulation in the thyroid of the living salamander that his interest became concentrated on the behavior of cells

and tissues within the living animal. This interest was the basis for Glenn's scientific work during his whole ensuing career. He came to the National Cancer Institute as Guest Worker, under a fellowship of the Finney-Howell Research Foundation, and at once undertook the problem of attempting to work out methods for studying normal and cancer cells implanted within the living mouse.

At this time, while others had already been able to insert "windows" into the ears of rabbits, through which living cells could be watched, the problem of inserting such observation windows into the mouse was beset with most discouraging difficulties. At the end of nearly two years' work, at a time when his fellowship was almost running out, Glenn made a "breakthrough" on this problem. He developed a method for insertion of transparent chambers into the skin of mice. This was so successful that by the use of such chambers he was able through the ensuing years to build up a group of highly precise methods and to carry out detailed studies on living normal and cancer cells within the animal. He developed quantitative methods for following rates of vascularization of implants and for measuring the pressure in blood vessels supplying the implants. He developed methods for observing individual cells and for following the reactions of cells and tissues by means of dramatic time-lapse pictures. In addition to the transparent observation chamber, he elaborated chambers made with graded porosity membranes so that cells and tissues implanted into such chambers were bathed by host tissue fluid although entirely separated from cells of the host. Both the methods developed by him and his many studies on cells and tissues in these "*in vivo* tissue cultures," as he often called them, have been a foundation for the work of numerous other scientists in this country and abroad.

In 1943 Glenn became a National Cancer Institute Research Fellow and soon after, Assistant Surgeon in the Commissioned Corps of the United States Public Health Service, continuing his work at the National Cancer Institute. He rose steadily in rank, being appointed Medical Director in 1957. He continued in this rank until his illness forced retirement just a short time before he died.

In 1940 Glenn married Dorothy Hamilton whom he had met in the laboratories at Woods Hole. Their three children, Anne, Thomas, and Stephen, are now 16, 14, and 12 years old, respectively. Glenn's whole homelife was a source of great and continuing pleasure and satisfaction to him. To an unusual degree he was absorbed by these interests, including the projects and the development of the children.

Glenn was a person with many interests. He read avidly and in widely varied fields. He became increasingly fond of music, and as the children grew old enough to study music, he himself started piano lessons. After marriage, through contact with Mr. George Hamilton, his father-in-law, he developed an intense and increasing interest in art, and during the later years of his life actually worked in oil painting. His bearing was quiet, pleasant, thoughtful, tinged by an ever-present trace of quiet humor. However, where a critical conclusion had to be reached, he could be decisive, or where a carefully considered ethical principle was involved, he could be both persistent and courageous.

By the reflections from these varied facets of his personality we remember him.

Wilton R. Earle

Publications of Glenn Horner Algire

1943–1946

A technique for the cytological study of the living thyroid gland. (With E. Uhlenhuth.) *J. Morphol. 75: 61–75.* 1944.

Colloid content and cell height as related to the secretory activity of the thyroid gland. I. In normal thyroids of *Triturus torosus.* (With E. Uhlenhuth, J. E. Schenthal, J. U. Thompson, and K. F. Mech.) *J. Morphol. 76: 1–29.* 1945.

An adaptation of the transparent-chamber technique to the mouse. *J. Nat. Cancer Inst. 4: 1–11.* 1943.

Microscopic studies of the early growth of a transplantable melanoma of the mouse, using the transparent-chamber technique. *J. Nat. Cancer Inst. 4: 13–20.* 1943.

Comparative oxidase activity of melanotic and amelanotic melanomas. (With J. P. Greenstein.) *J. Nat. Cancer Inst. 5: 35–38.* 1944.

Growth and pathology of melanoma S91 in mice of strain dba, A, and C. *J. Nat. Cancer Inst. 5: 151–160.* 1944.

The vascular supply of mammary gland carcinomas. *In* Moulton, A Symposium on Mammary Tumors in Mice. *Washington, D. C., Am. Assoc. Advancement Sc., Publ. No. 22, pp. 47–54.* 1945.

Vascular reactions of normal and malignant tissues *in vivo.* I. Vascular reactions of mice to wounds and to normal and neoplastic transplants. (With H. W. Chalkley.) *J. Nat. Cancer Inst. 6: 73–85.* 1945.

Effect of the level of dietary protein on vascular repair in wounds. (With H. W. Chalkley.) *J. Nat. Cancer Inst. 6: 363–372.* 1946.

1947–1950

The transparent-chamber technique as a tool in experimental tumor therapy. *In* Moulton, Approaches to Tumor Chemotherapy. *Washington, D. C., Am. Assoc. Advancement Sc., pp. 13–26.* 1947.

Characterization of tissue metabolism of transplanted mouse melanomas by high oxidative response to paraphenylenediamine. (With D. Burk, M. L. Hesselbach, C. E. Fischer, and F. Y. Legallais.) *J. Nat Cancer Inst. 7: 425–429.* 1947.

Vascular reactions of normal and malignant tissues *in vivo.* II. The vascular reaction of normal and neoplastic tissues of mice to a bacterial polysaccharide from *Serratia marcescens* (*Bacillus prodigiosus*) culture filtrates. (With F. Y. Legallais and H. D. Park.) *J. Nat. Cancer Inst. 8: 53–62.* 1947.

Growth and vascularization of transplanted mouse melanomas. (With F. Y. Legallais.) *In* The Biology of Melanomas. *Special Publ. New York Acad. Sc. 4: 159–171.* 1948.

Tissue metabolism of transplanted mouse melanomas with special reference to characterization by paraphenylenediamine. (With D. Burk, M. L. Hesselbach, C. E. Fischer, and F. Y. Legallais.) *In* The Biology of Melanomas. *Special Publ. New York Acad. Sc. 4: 437–446.* 1948.

Recent developments in the transparent-chamber technique as adapted to the mouse. (With F. Y. Legallais.) *J. Nat. Cancer Inst. 10: 225–253.* 1949.

The effect of vascular occlusion on transplanted tumors. (With J. S. Youngner.) *J. Nat. Cancer Inst. 10: 565–580.* 1949.

Circulatory reactions in photodynamic action. (With J. U. Schlegel.) *J. Cell. & Comp. Physiol. 35: 95–110.* 1950.

Transparent-chamber observations of the response of a transplantable mouse mammary tumor to local roentgen irradiation. (With R. Merwin and H. S. Kaplan.) *J. Nat. Cancer Inst. 11: 593–623.* 1950.

Vascular reactions of normal and malignant tissues *in vivo.* III. Vascular reactions of mice to fibroblasts treated *in vitro* with methylcholanthrene. (With W. R. Earle and H. W. Chalkley.) *J. Nat. Cancer Inst. 11: 555–579.* 1950.

1951–1953

Vascular reactions of normal and malignant tissues *in vivo*. IV. The effect of peripheral hypotension on transplanted tumors. (With F. Y. Legallais.) *J. Nat. Cancer Inst. 12: 399–421.* 1951.

Vascular reactions of normal and malignant tissues *in vivo*. V. The role of hypotension in the action of a bacterial polysaccharide on tumors. (With F. Y. Legallais and B. F. Anderson.) *J. Nat. Cancer Inst. 12: 1279–1295.* 1952.

Microscopic studies on tumor histo-physiology in transparent chambers. (With F. Y. Legallais.) *Acta Unio internat. contra cancrum. 11: 759–760.* 1952.

Transparent-chamber techniques. *In* Cowdry, Laboratory Technique in Biology and Medicine, 3rd edition. Baltimore, Williams & Wilkens, *pp. 354–356.* 1952.

Growth and histopathology of melanotic and amelanotic derivatives of the Cloudman melanoma S91. (With P. Loustalot, F. Y. Legallais, and B. F. Anderson.) *J. Nat. Cancer Inst. 12: 1079–1117.* 1952.

Growth and pathology of melanotic and amelanotic derivatives of the Cloudman melanoma S91. (With P. Loustalot, F. Y. Legallais, and B. F. Anderson.) *In* Pigment Cell Growth. *New York, Academic Press, pp. 93–99.* 1953.

1954–1955

The transparent-chamber technique for observation of the peripheral circulation as studied in mice. *In* J. and A. Churchill, Peripheral Circulation in Man. *London, Publ. Ciba Foundation, pp. 58–67.* 1954.

Determination of peripheral blood pressure in unanesthetized mice during microscopic observation of blood vessels. *J. Nat. Cancer Inst. 14: 865–877.* 1954.

Visualizing cellular behavior "*in vivo*." *In XV Congres de la Societe Internationale de Chirurgie: Bruxelles, Imprimerie Henri de Smedt, pp. 1–11.* 1954.

Vascular reactions of normal and malignant tissues *in vivo*. VI. The role of hypotension in the action of components of podophyllin on transplanted sarcomas. (With F. Y. Legallais and B. F. Anderson.) *J. Nat. Cancer Inst. 14: 879–893.* 1954.

Vascular reactions of normal and malignant tissues *in vivo*. VII. Observations on vascular reactions in destruction of tumor homografts. *J. Nat. Cancer Inst. 15: 483–491.* 1954.

Growth of cells *in vivo* in diffusion chambers. I. Survival of homografts in immunized mice. (With J. M. Weaver and R. T. Prehn.) *J. Nat. Cancer Inst. 15: 493–507.* 1954.

The diffusion-chamber technique applied to a study of the nature of homograft resistance. (With R. T. Prehn and J. M. Weaver.) *J. Nat. Cancer Inst. 15: 509–517.* 1954.

The growth of cells *in vivo* in diffusion chambers. II. The role of cells in the destruction of homografts in mice. (With J. M. Weaver and R. T. Prehn.) *J. Nat. Cancer Inst. 15: 1737–1767.* 1955.

Vascular patterns in tissues and grafts within transparent chambers in mice. (With R. M. Merwin.) *Angiology 6: 311–320.* 1955.

1956–1958

The role of graft and host vessels in the vascularization of grafts of normal and neoplastic tissue. (With R. M. Merwin.) *J. Nat. Cancer Inst. 17: 23–33.* 1956.

In vivo cell culture. *Univ. Michigan Bull. 22: 517–523.* 1956.

Studies on tissue homotransplantation in mice using the diffusion-chamber methods. (With J. M. Weaver and R. T. Prehn.) *Ann. New York Acad. Sc. 64: 1009–1012.* 1957.

Diffusion-chamber techniques for studies of cellular immunity. *Ann. New York Acad. Sc. 69: 663–667.* 1957.

Summary of studies of transplantation of homologous tissues. *Federation Proc. 16: 601–602.* 1957.

Studies of homographs using transparent and diffusion chamber methods. *Colloque Internationeux du Centre National de le Recherche Scientifique.* In press.

Studies of heterografts in diffusion chambers in mice. (With M. L. Borders and V. J. Evans.) *J. Nat. Cancer Inst. 20: 1187–1201.* 1958.

Effect on the Medium of Growth of Clone NCTC 929 Cells as Suspensions in Agitated Flasks With Simple Fluid Addition [1]

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Summary

To test the practicality of simple fluid addition instead of fluid change, the cells from clone NCTC 929 of strain L of mouse fibroblast origin were grown in agitated suspension with continuous gas exchange. Increments of fresh medium were added to the used medium at stated intervals. In this fashion the volume of the supporting fluid was gradually increased with opportunity for accumulation in the medium of metabolic products from the cells. During 10 days the cells with the higher fluid increment and approximately 330 million cells for the inoculum grew up to 2.5× the inoculum. The 2 cultures getting the lower increment, but started with about 90 million cells, grew during 12 days up to 7× the inoculum. The populations of all 4 cultures then rapidly declined. A moderate amount of glucose was removed from the medium with comparatively little of it accumulating as lactic acid. Total free amino acids of the medium were increased after use of the medium by the cells. Only certain ones were responsible for this increase. So far as one may conclude from this series of experiments, simple fluid addition would not be a suitable procedure for long-term culture of the cells in agitated suspensions. **J. Nat. Cancer Inst. 21: 429–435, 1958.**

Clone NCTC 929 of strain L of mouse fibroblast cells is cultured without difficulty [3] in shaker flasks or agitated medium (*1–5*) with continuous gas exchange (*6, 7*). It would greatly simplify handling of massive quantities of the cells if freshly made medium could be added periodically to the medium in which the cells subsist. This simple fluid addition would be in contrast to the usual complete fluid renewal at given intervals. The usual fluid renewal involves removal of the old fluid and replacement with either an aliquot of the original medium, kept sterile in the interim, or with

[1] Received for publication September 26, 1957.

[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

[3] There is some difficulty, however, in comparing results from experiment to experiment in the published work of various investigators. Different mediums were used. Many experiments were run without continuous gassing. Adjustment of initial *p*H was variable. Original inoculums covered a range of several hundredfold.

newly made medium. In any study of the practicality of such a procedure it is desirable to have some knowledge of the changes the cells have made in the medium by their use of it. The part of the study presented here included the estimation of the glucose, pyruvic acid, urea, glutamine, ammonia, α-ketoglutaric acid, and free amino acids of the 3 pertinent fluids; namely: the freshly prepared medium, aliquots of medium incubated under the same experimental conditions as those added to the cells but with no cells present, and the used medium collected as supernatant fluid after centrifugal removal of the cells.

Exploratory studies have been made on the effect of different levels of fluid increment added to measured initial volume of the medium with the cells in suspension culture (*5*, *6*). From these experiments the increments of 35 and 65 ml. were chosen for further study, and it is with 4 of these studies that the present report is concerned. The studies covered a 14-day interval.

Experiments and Results

Cells from the clone 929 mouse fibroblast strain were grown in agitated fluid-suspension culture in shaker flasks, with continuous gassing, as described elsewhere (*7*). After the initial suspension of the cells in the medium, fluid was added periodically as indicated at the rate of 35 ml. to each of two flasks, 1 and 2, and at the rate of 65 ml. to the others, 3 and 4, per given time interval. In this fashion the cells continued in the old medium with a constant volume but decreasing percentile increment of fresh fluid. Thus for the 65 ml. increment the first addition represented a 29 percent increase; the last was only 14 percent. A fifth flask contained only shaken medium incubated and gassed as a control. Fluid increments were put in at the upper level, *i.e.*, 65 ml., and into all flasks at the third, fifth, seventh, tenth, and twelfth days following the initial suspension of the cells in 150 ml. The population at each interval was ascertained by counting cell nuclei (*8*). Two ml. samples of the suspensions were withdrawn for cell count and glucose estimation immediately before fluid increment addition. An effort was made to return the glucose level at each increment to more than 100 mg. per 100 ml. of the suspensions. This was accomplished in all instances except for the tenth day of experiment 3 when the level reached was only 62 mg. Glucose,[4] protein, lactic acid, keto acids, urea, ammonia, and the free amino acids were estimated as previously described (*11–13*). The individual amino acids were separated by paper chromatography and the quantities ascertained by means of a densitometer. The medium used for growth of the cells was made up of horse serum, saline, and the protein-free extract of the hen's egg. These constituents were mixed in the proportions of 40:40:20 percent by volume. The extract had been made by treating the freshly laid

[4] Glucose was estimated by using the modified Sumner method (*9*) on the initial medium and on aliquots of the terminal medium pertinent to each culture. For independent estimations on an aliquot at the time of each fluid addition for each flask, we are indebted to Dr. W. C. Alford of the Institute of Arthritis and Metabolic Diseases. His estimations were by the Nelson-Somogyi (*10*) procedure. Results agreed within 6 percent, with the number 4 culture yielding identical values for total glucose used by the 2 procedures.

hen's egg with 1.25× its volume of balanced saline [5] and ultrafiltering the extract to remove the large molecular portion (*15*). The cell suspensions initially were prepared in 150 ml. of the nutrient fluid. For the 4 experiments the inoculums in millions were as follows: #1, 95; #2, 88; #3, 329; #4, 348. The starting medium per 100 ml. had in it 3 mg. N as free amino acids, amides, and amines; the horse serum furnished 450 mg. N as protein; urea represented 5 mg. N. The urea was almost entirely derived from the horse serum; very little urea was found in the whole egg extract (*13*). The total free amino acids furnished 14 mg. N to the cells by the time the samples were taken for final analysis, and the total protein N available from the horse serum of the medium was 1930 mg. N.

Results from the investigation are shown in tables 1 through 5. Table 1 has the cell populations, fluid volumes, and glucose supplied and used by

TABLE 1.—*Volume of fluid suspensions, populations, and glucose concentrations during time course of experiments 1 and 2; 35 ml. fluid increments*

Day	Fluid volume (*ml.*)		Cells (*millions*)	Glucose (*mg./100 ml.*)	
	Old	New		Old	New
Experiment 1					
0	—	150	95	—	380
3	150	183	194	237	255
5	183	216	321	223	234
7	216	249	361	206	224
10	249	282	459	183	186
12	282	315	697	171	174
14	315	—	190	150	—
Experiment 2					
0	—	150	88	—	380
3	150	183	165	237	299
5	183	216	296	237	235
7	216	249	347	203	222
10	249	282	402	172	178
12	282	315	607	156	163
14	315	—	209	139	—

the cultures getting the smaller medium increment. Similar data for the 2 cultures getting the larger increment are shown in table 2. Table 3 has the glucose consumption expressed on a unit cell basis. Cultures 3 and 4 with the larger inoculum were considered to have greater interest for possible changes in the medium. The medium for each of these was examined for other constituents shown in tables 4 and 5. Cells from cultures 1 and 2 were taken for certain enzymic studies and for study of the composition of the cells. Some aspects of the examination will be discussed.

[5] Saline here refers to the balanced saline containing bicarbonate routinely used in this laboratory (*14*).

TABLE 2.—*Volume of fluid suspensions, populations, and glucose concentrations during time course of experiments 3 and 4; 65 ml. fluid increments*

Day	Fluid volume (ml.)		Cells (millions)	Glucose (mg./100 ml.)	
	Old	New		Old	New
Experiment 3					
0	—	150	329	—	380
3	150	213	778	111	176
5	213	276	798	87	114
7	276	339	884	69	122
10	339	402	882	36	62
12	402	465	380	32	138
14	465	—	503	86	—
Experiment 4					
0	—	150	348	—	380
3	150	213	398	160	210
5	213	276	617	167	204
7	276	339	592	125	164
10	339	402	813	94	111
12	402	465	466	60	164
14	465	—	497	130	—

TABLE 3.—*Micromoles glucose used per 100 million cells per hour during experimental period for each culture*

Expt. No.	1	2	3	4
Micromoles	28	34	31	38

TABLE 4.—*Effect of growth of strain 929 cells on lactic acid, pyruvic acid, α-ketoglutaric acid, and total free amino acids of the two experiments 3 and 4 getting the greater fluid increment during culture in agitated suspensions. Total milligrams per culture*

Item	Experiment No.	
	3	4
Lactic acid added	30	30
Excess lactic acid formed	30	280
Pyruvic acid added	2.3	2.3
Change in pyruvic acid	−1.6	+3.3
α-Ketoglutaric acid added	0.9	0.9
Change in α-ketoglutaric acid	−0.9	+1.4
Free amino acid N added	14	14
Change in free amino acid N	+4.5	+4.0

TABLE 5.—*Concentration of urea, ammonia, and certain free amino acids in medium before and after growth of cells*

Amino acid	Fresh medium (*mg.*)	Expt. 3 (*mg.*)	Expt. 4 (*mg.*)
Alanine	1. 6	4. 0	4. 0
Arginine	0. 8	0. 6	0. 6
Cystine (cysteine)	0. 6	0. 2	0. 3
Glutamic acid	0. 4	3. 0	3. 0
Glutamine	4. 5	4. 0	4. 0
Glycine-serine	1. 3	2. 2	2. 2
Histidine	0. 6	0. 3	0. 3
Isoleucine-leucine	1. 5	0. 3	0. 3
Lysine	1. 4	trace	trace
Methionine	0. 2	0. 4	0. 4
Phenylalanine	0. 4	0. 3	0. 3
Proline	1. 2	1. 2	1. 3
Threonine	0. 7	1. 0	1. 0
Tyrosine	0. 5	trace	trace
Tryptophan	1. 6	trace	trace
Valine	1. 0	0. 5	0. 5
Unidentified	2. 5	8. 5	8. 5
Urea N*	5. 0	5. 2	5. 4
Ammonia N	0. 1	0. 8	1. 2

*The Archibald procedure required that a correction be made for phenol red in all mediums. The differences in the 3 values for urea are barely beyond the error of the method in which 10 replicate estimations were set up on each medium.

Discussion

In experiments 1 and 2, the inoculums for the 2 cultures were around 600,000 cells per ml., and those for cultures 3 and 4, getting the higher increment, were more than 3.5× this figure. In all 4 experiments the culture populations increased for some days and then broke back sharply. Why this occurred is obscure. Since this work was completed, Thomas *et al.* (*7*) have reported that simple addition of arginine to Spinner (*2*) cultures protected against this breakback in the populations. However, the figures they cited showed evidence that the nature of the phenomenon was not completely resolved since the culture populations fell off at about the same time and population-point as in this investigation. In the consideration of some of the possible injurious factors in the suspensions under the circumstances of these experiments, the accumulation of precipitating protein in the medium is discussed. McLimans reports that in the Spinner cultures there is no proteinaceous precipitate, so that for his cultures no such effect could be invoked. The precipitating protein in the medium of the present experiment is independent of the presence of the cells because the shaken and gassed acellular medium likewise accumulated this protein. It was thrown down with the cells when they were lightly packed in the bottom of a suitable centrifuge tube to remove them from the medium. Total dry weight of the cells, total protein, lipide, etc., cannot be assayed properly under these circumstances. In calculation of the cell mass it had to be assumed that the precipitate in the shaken medium occurred in equivalent concentration to that in the experimental flasks. When the precipitated protein alone was taken into account, *i.e.*,

subtracted from the total mass, the resulting wet weight values of cells from the 1 and 2 cultures were 1.5 mg. per million cells. The 1.5 mg. is considerably less than that obtained from the cells of this strain grown in still cultures in the T-60 flasks in stock medium (*16*). It is, however, greater than the weight found by Oyama and Eagle in a different medium (*17*). Cells in the agitated cultures show a decided apparent rounding-up compared to their appearance in still cultures as observed not only in this medium but also by Siminovitch *et al.* in a different medium (*1*). The rounding-up may give a smaller and much more compact cell.

As shown in table 3 the glucose utilization per cell was much the same for the 4 cultures. In no instance was there exhaustion of the glucose in the medium, so that glucose was not a limiting factor for the cell growth. As indicated in table 4 there was an increase in total free amino acids and ammonia in all of the culture flasks with cells present. Increase in certain of them, such as alanine and glutamic acid, is now a well-known phenomenon (*18–20*) and is probably to be ascribed to transamination. Table 5 indicates that certain amino acids may have reached concentrations that were too low to support continuous growth at the high levels of the earlier portions of the period. It is known that in the absence of certain essential amino acids (*1, 21*) or amines, the L strain of cells or its derived strain 2071 (*22*) cannot grow and cannot use glucose even though it is available. The accumulation of 10× the concentration of ammonia in the used medium compared to the acellular medium may be an additional deleterious factor. The ammonia that accumulates in the used medium during growth of the cells of various origins, both in shaker and agitated cultures, is believed to be very largely derived from the glutamine (*20, 22, 23*) and is observed in still cultures (*20, 22*), so that it may not be considered peculiar to cultures under agitated conditions or to the accumulating used fluid conditions of the present study. So far as one may draw inferences from the study, it would appear that the increment addition as carried out here has little practicality for the continuous growth of the cells. Results might have been better with a constant percentile increment instead of the constant volume increment as added here. However, the population data for the first and second cultures indicate that for a 10-day interval the procedure could be used to obtain as much as a sevenfold increase in populations and a total quantity of 700 million cells.

References

(*1*) Siminovitch, L., Graham, A. F., Lesley, S. M., and Nevill, A.: Propagation of L strain mouse cells in suspension. Exper. Cell Res. 12: 299–308, 1957.

(*2*) McLimans, W. M., Davis, E. V., Glover, F. L., and Rake, G. W.: The submerged culture of mammalian cells: The Spinner culture. J. Immunol. 79: 428–433, 1957.

(*3*) Kuchler, R. J., and Merchant, D. J.: Propagation of strain L (Earle) cells in agitated fluid suspension cultures. Proc. Soc. Exper. Biol. & Med. 92: 803–806, 1956.

(*4*) Earle, W. R., Bryant, J. C., and Schilling, E. L.: Certain factors limiting the size of the tissue culture and the development of massive cultures. Ann. New York Acad. Sc. 58: 1000–1011, 1954.

(5) EARLE, W. R., BRYANT, J. C., SCHILLING, E. L., and EVANS, V. J.: Growth of cell suspensions in tissue culture. Ann. New York Acad. Sc. 63:666–682, 1956.

(6) BRYANT, J. C., SCHILLING, E. L., EARLE, W. R., and EVANS, V. J.: Massive fluid-suspension cultures of certain mammalian tissue cells. II. Glucose utilization and cell proliferation. J. Nat. Cancer Inst. 21: 349–364, 1958.

(7) THOMAS, W. J., ZIEGLER, D. W., SCHEPARTZ, S. A., and MCLIMANS, W. F.: Use of arginine to eliminate medium changes in tissue culture systems. Science 127: 591–592, 1958.

(8) SANFORD, K. K., EARLE, W. R., EVANS, V. J., WALTZ, H. K., and SHANNON, J. E., JR.: The measurement of proliferation of tissue cultures by enumeration of cell nuclei. J. Nat. Cancer Inst. 11: 773–795, 1951.

(9) HENDRIX, J. P., WESTFALL, B. B., and RICHARDS, A. N.: Quantitative studies of the composition of glomerular urine. XIV. The glomerular excretion of inulin in frogs and necturi. J. Biol. Chem. 116: 735–747, 1936.

(10) NELSON, N.: A photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem. 153: 375–380, 1944.

(11) WESTFALL, B. B., PEPPERS, E. V., SANFORD, K. K., and EARLE, W. R.: The amino acid content of the ultrafiltrate from horse serum. J. Nat. Cancer Inst. 15: 27–35, 1954.

(12) WESTFALL, B. B., PEPPERS, E. V., and EARLE, W. R.: α-Keto acids in tissue culture. I. The concentration of pyruvic and α-ketoglutaric acids in the ultrafiltrates, from horse serum and chick-embryo extract. J. Nat. Cancer Inst. 16: 337–347, 1955.

(13) ———: The change in concentration of certain constituents of the medium during growth of the strain HeLa cells. Am. J. Hyg. 61: 326–333, 1955.

(14) EARLE, W. R., SCHILLING, E. L., STARK, T. H., STRAUS, N. P., BROWN, M. F., and SHELTON, E.: Production of malignancy *in vitro*. IV. The mouse fibroblast and changes seen in the living cells. J. Nat. Cancer Inst. 4: 165–212, 1943.

(15) EVANS, V. J., BRYANT, J. C., MCQUILKIN, W. T., FIORAMONTI, M. C., SANFORD, K. K., WESTFALL, B. B., and EARLE, W. R.: Studies of nutrient media for tissue cells *in vitro*. III. Whole-egg extract ultrafiltrate for long-term cultivation of strain L cells. Cancer Res. 17: 317–324, 1957.

(16) WESTFALL, B. B., EVANS, V. J., SHANNON, J. E., JR., and EARLE, W. R.: The glycogen content of cell suspensions prepared from massive tissue culture: Comparison of cells derived from mouse connective tissue and mouse liver. J. Nat. Cancer Inst. 14: 655–664, 1953.

(17) OYAMA, V. I., and EAGLE, H.: Measurement of cell growth in tissue culture with a phenol reagent (Folin-Ciocalteau). Proc. Soc. Exper. Biol. & Med. 91: 305–307, 1956.

(18) MARKERT, C. L.: Some trends in research on cellular differentiation and morphogenesis. Discussion. J. Nat. Cancer Inst. 19: 632–635, 1957.

(19) PASIEKA, A. E., MORTON, H. J., and MORGAN, J. F.: The metabolism of animal tissues cultivated *in vitro*. I. Amino acid metabolism of chick embryonic heart fibroblasts cultivated in synthetic medium M 150. J. Nat. Cancer Inst. 16: 995–1003, 1956.

(20) WESTFALL, B. B., EVANS, V. J., PEPPERS, E. V., HAWKINS, N. M., and EARLE, W. R.: Effect of glutamine on the growth and metabolism of liver cells *in vitro*. J. Nat. Cancer Inst. 17: 131–138, 1956.

(21) EAGLE, H.: The specific amino acid requirements of a mammalian cell (strain L) in tissue culture. J. Biol. Chem. 214: 839–852, 1955.

(22) PEPPERS, E. V., FIORAMONTI, M. C., WESTFALL, B. B., EVANS, V. J., and EARLE, W. R.: Effect of lack of glutamine on subline 2071 mouse cells. J. Nat. Cancer Inst. 21: 611–620, 1958.

(23) LEVINTOW, L., EAGLE, H., and PIEZ, K. A.: The role of glutamine in protein biosynthesis in tissue culture. J. Biol. Chem. 227: 929–941, 1957.

Mammalian Chromosomes *in Vitro*. IX. On Genetic Polymorphism in Cell Populations [1, 2]

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Summary

Five cell strains MC3, CF1-C-9, NCTC 2071 (NCTC clone 929, strain L), L-P55, and L-P55-N2, all of murine origin, were studied with respect to their chromosomal constitution. The last 3 strains were sublines of NCTC clone 929 (strain L). All of them were heteroploid. Besides the changes in chromosome number, each strain possessed from 10 to 20 metacentrics and a few subtelocentric chromosomes. Each strain had its characteristic karyotype and was cytologically identifiable. Cells of strains L-P55 and L-P55-N2 contained 1 or 2 long dicentric chromosomes. However, a small proportion of the cells in strain L-P55 was void of this element. This dicentric chromosome was probably identical to the dicentric that occurred frequently in strain L_2 established by Fedoroff in Saskatoon, Canada. Within each strain considerable variations of chromosome number as well as combination of chromosome types were recorded. Therefore, each strain is heterogeneous in its genetic make-up. Genetic polymorphism conceivably prevails also in populations of other cell strains. It provides different genotypes for the populations to adjust themselves to various adverse selection forces that may be applied to them.
J. Nat. Cancer Inst. 21: 437–473, 1958.

Cells of both normal and malignant origin, after continuous cultivation *in vitro*, invariably exhibit extensive heteroploidy (*1–4*). In neoplasms where heteroploids may have originally dominated the *in situ* tissues, heteroploidy found in the *in vitro* cells may not indicate a drastic change; but in nonmalignant tissues, the process of transformation from diploid to heteroploid has intrigued both biologists and medical researchers because of its numerous theoretical and practical implications. In a recent paper (*5*) such transformation was witnessed in one human tissue where all the

[1] Received for publication December 9, 1957.

[2] Supported in part by grant P-133 from the American Cancer Society, Inc., and by grant DRG-269C from the Damon Runyon Memorial Fund for Cancer Research, Inc.

[3] The authors are grateful to Dr. Albert Levan for his many important criticisms. Valuable technical assistance was provided by Mrs. Lillian Milofsky and Miss Sally J. Gardner.

cells in the strain became secondary heteroploid in approximately 4 months. Hsu (*6*) has presented some perspective discussions on the problem of chromosomal changes in relation to cell metabolism, environmental adaptation, and carcinogenesis.

When one takes any such cell strain for cytological examination, he readily finds that, although the cells are all heteroploid, they differ in chromosome numbers. Variation of chromosome numbers occurs even in some *in vivo* populations, such as the cystocarcinoma of the ovary (*7*), in which at least 15 or 16 chromosome numbers are found. If each chromosome number represents one genotype, the population already contains 15 or 16 genotypes. Now, are the cells of the same chromosome number genetically identical? A number of them are expected to be so because regular mitoses occur; but conceivably not all of them are identical. The possibility of a combination of chromosomes reaching the same heteroploid number is unlimited.

In order to prove the existence of genetic variability among cells in cell lines, several methods are available. Comparative study on metabolic patterns of substrains derived from the same source would be the most direct way of producing evidence for genetic heterogeneity of the populations. However, it is relatively difficult to obtain criteria for such a demonstration. The difference in the so-called "plating efficiency" between clones S1 and S3 of strain HeLa (*8*) may be a reflection of their metabolic differences. An alternative method is to utilize clone strains for detection of cytological differences. Chu and Giles (*9*) studied several clones of HeLa and found that, with the exception of 2 that had identical chromosome number, each clone showed a characteristic modal number of its own. The third method is the karyogrammatic analysis of chromosomal variability in nonpurified populations that would also provide evidence for the phenomenon of genetic heterogeneity. The last method is probably the safest because, in the process of cloning, certain more fastidious (genetically inferior?) cells that might survive in mixed populations may be unable to maintain themselves as clones, thus lowering the observed range of variation.

The present article reports the analysis of chromosome numbers and morphology in several cell strains of murine origin as examples to elucidate the phenomenon of genetic polymorphism in cell populations.

Materials and Methods

Strains MC3 and CF1-C-9 were established by McCulloch and Parker (*10*) from the bone marrow of C57BL and CF_1 mice, respectively. Our cultures were supplied through the kindness of Dr. Daniel Billen, Section of Microbiology, The University of Texas M. D. Anderson Hospital and Tumor Institute, who originally had obtained the stocks from Dr. E. A. McCulloch. Strain NCTC 2071, a subline of NCTC clone 929 (strain L), was kindly sent to us by Dr. Wilton R. Earle, National Cancer Institute. The origin of the strains L-P55 and its substrain L-P55-N2 will be described in their respective sections. The cell lines have been carried

routinely in our laboratories in Eagle's basic medium with 10 percent horse serum.

In compliance with a number of requests for the techniques of preparing slides for chromosome studies from tissue-culture materials, we present detailed accounts of our procedure. The scheme is based on the methods used by Levan (*3*) and Ford and Hamerton (*11*), with some modifications.

1) Select cultures at their peak of mitotic activity (the log phase of growth), generally between 2 to 7 days after subculturing. Prepare a 1:10,000 solution of colchicine, with either sterile water or balanced salt solution as diluent. This solution can be stored in the refrigerator indefinitely. Add 0.2 ml. of this solution to each of 10 ml. of growth medium, allowing the cells to be treated for 16 to 18 hours. This treatment arrests most of the mitotic cells at metaphase yet is not long enough to allow any mitotic cell to enter its next division, which would falsify the picture for the proportion of polyploids in the population. Colchicine also condenses the chromosomes considerably so that the counts can be more accurate.

2) At the end of the treated period, suspend the cells with a rubber policeman, and centrifuge the cells. Treat the cells with a hypotonic solution for approximately 10 minutes. The hypotonic solution can be prepared either by mixing 2 parts of balanced salt solution and 8 parts of tap water or by preparing a 1.12 percent sodium citrate in distilled water. The cells should be resuspended during this treatment to ensure maximum swelling.

3) Centrifuge the cells again and pour off the hypotonic solution. Fix the cells with freshly prepared acetic alcohol. No stirring of cells is needed, but the material should be left in this mixture for at least 20 minutes. The cells can either be preserved in this fixative or changed to 70 percent alcohol for storage. The safest way is to squash a part of the freshly fixed cells and preserve the remainder.

4) Replace the fixative or preservative with approximately 2 ml. of 50 percent glacial acetic acid and add 1 or 2 drops of 2 percent acetic orcein to the container. The procedure of adding stain to acetic acid is not absolutely necessary. Use a Pasteur pipette to break up the cell clusters. Place 1 small drop of the cell suspension on a clean slide and put a coverslip (preferably 22 × 30 mm. rectangular) over the suspension. There should not be an excess of fluid at the edges of the coverslip. Use a blotting paper to cover the slide, and press the coverslip with the thumb. The pressure of the thumb should be so adjusted that *maximum flattening of the cells with minimum amount of breakage* is obtained. Use a phase-contrast microscope at low magnification for inspection.

5) Seal the preparation just squashed with Deckglaskit nach Krönig.[4] The temporary preparations, if sealed airtight, can be stored at room temperature for several months so that the investigators do not feel pressed to rush the reading. Since little stain is used in our procedure, aging of the slides hardly deteriorates the quality of the preparations.

6) Use a phase-contrast microscope for examination. The staining is so light that an ordinary light microscope reveals practically nothing. With a phase microscope the chromosomes show as much contrast as those heavily stained and observed with a light microscope, and the cytoplasm shows a low but definite contrast compared with the background. This is very important because the intactness of the cells can be assured only by the intactness of the cytoplasmic outline.

In the present study only the cells with complete outline and good spread of chromosomes were used. Fifty cells were carefully examined and recorded for each set. Camera-lucida drawings were made by placing each chromosome at the center of the viewfield with a 100× objective and a 25× ocular.

[4] This wax can be purchased from Riedel-de Haën A.-G., Seelze bei Hannover, Germany (minimum order, $10.00).

Sources of Error

With the techniques described in the previous section, there is no difficulty in finding the desired number of cells with their chromosomes so perfectly spread that the counting error can be minimized to practically zero in most cases. Occasionally, however, a biarmed chromosome with a far-stretched centromeric zone may be mistaken for two. The main source of error could come from incomplete cells where a piece of cytoplasm carrying a few chromosomes broke away, thus giving a count lower than it should have been. With the aid of a phase microscope, incomplete cellular outline can be detected with ease and anytime such a cell appears it should be discarded for counting purposes. However, there is a possibility that a broken piece could be from the top or the bottom part of a cell; the cellular outline would appear intact but the count would then be false. The exact number of such cells included in our data is unknown.

In studying the chromosome morphology the error of misdetermination of a special chromosome would not be far greater than that of the counts because of the excellent spread of the chromosomes on a relatively flat plane. The main difficulty in karyotypic investigations was the differentiation of telocentric and nearly telocentric chromosomes. On a number of these chromosomes a small, knoblike structure could be seen as if it were a minute second arm. In some cells, nearly all the "telocentric" chromosomes appeared to bear such knobs; in others, none. Using the chromosomes of normal mouse cells (all telocentric) for comparison, we found that they exhibited a similar feature. Perhaps some of the telocentric chromosomes of the cell strains studied did possess "bona fide" second arms that should have been classified as subtelocentrics; but for the sake of lessening our error, we henceforth group all these elements under the heading "telocentrics."

Another delicate task in our determination of chromosomes was to distinguish submetacentrics and subtelocentrics (subterminals), for they showed a continuous variation of the arm-length ratio. We adopted an arbitrary standard for our grouping of chromosomes into submetacentric and subtelocentric classes: when one arm of a chromosome was half the length of the other or longer, the chromosome was regarded as submetacentric. Otherwise it was regarded as subtelocentric. Fortunately, such dubious cases were not too often encountered.

Chromosomes of Strain MC3

Strain MC3 was totally heteroploid with a rather narrow range (75 to 82) of distribution of chromosome numbers. The majority of cells exhibited 78 to 80 chromosomes. High polyploid elements constituted less than 1 percent of the population. Figures 1 and 2 represent 2 cells of this line, and figures 5 through 8 are ideograms of 4 randomly selected cells showing the metacentric and the subtelocentric chromosomes only. Table 1 presents the data on the distribution of chromosome types among 50 cells.

TABLE 1.—*Distribution of chromosome types in 50 cells of strain MC3—high polyploids not included; chromosome fragments not recorded*

Number of chromosomes	M*	S†	T‡	Number of cells
75	15	1	59	1
75	15	2	58	1
75	16	1	58	1
76	15	1	60	1
76	16	0	60	1
76	16	2	58	1
76	17	2	57	1
77	14	2	61	1
77	16	2	59	1
77	17	1	59	1
78	13	2	63	1
78	14	2	62	1
78	15	1	62	1
78	15	2	61	2
78	16	1	61	6
78	17	1	60	2
78	18	1	59	1
78	19	3	56	1
79	12	2	65	1
79	14	1	64	1
79	14	2	63	1
79	15	1	63	3
79	16	1	62	3
79	16	2	61	2
79	17	1	61	1
80	14	1	65	1
80	15	1	64	2
80	16	1	63	2
80	16	2	62	2
80	17	2	61	1
81	13	2	66	1
81	16	2	63	1
82	14	1	67	1
82	14	3	65	1
82	17	1	64	1
Average 78. 5	15. 5	1. 4	61. 6	

*Metacentrics and submetacentrics.
†Subtelocentrics.
‡Telocentrics.

There were 12 to 19 metacentric chromosomes per cell, with 14 to 17 the most common numbers. Among the metacentrics 1 was very short and some were actually submetacentrics with one arm distinctly longer than the other. One of the submetacentrics (occasionally absent) was the longest of the complement (figs. 5 and 7). In figure 8 a unique submetacentric chromosome, approximately 1½ times as long as any of the longest chromosomes, was found. Probably it was the result of a new translocation between a metacentric and another chromosome.

Out of 50 cells studied (table 1) only 1 cell contained no subtelocentric chromosome. In all the other 49 cells a long subtelocentric element consistently appeared. In a number of cells, 2 such chromosomes that appeared identical were recorded (fig. 6). Small subtelocentrics were also found in several cells (figs. 5, 6, and 8).

If we employ the conventional symbols D, M, S, and T to designate

dicentric, metacentric (including submetacentric), subtelocentric, and telocentric chromosomes, respectively, the karyological features of strain MC3 can be described in a simple formula, M15-16:S1:T61-63(78 to 80). The figures inside the parentheses represent the most common chromosome numbers of the strain.

Chromosomes of Strain CF1-C-9

Superficially the chromosomal characteristics of strain CF1-C-9 (figs. 3 and 4) were similar to those of strain MC3. However, a detailed analysis (table 2 and figs. 9 through 12) revealed a number of differences between these 2 lines. Most of the cells of strain CF1-C-9 contained 80 to 82 chromosomes. The average number of metacentric chromosomes per cell in strain CF1-C-9 was 14.6, 1 lower than that of MC3. The most significant feature of CF1-C-9 was that most of the cells contained 2 subtelo-

TABLE 2.—*Distribution of chromosome types in 50 cells of strain CF1-C-9—high polyploids not included; chromosome fragments not recorded.*

Number of chromosomes	M*	S†	T‡	Number of cells
68	13	2	53	1
76	15	2	59	1
77	13	2	62	1
77	15	1	61	1
78	13	2	63	1
78	15	2	61	1
79	13	1	65	1
79	14	2	63	3
79	15	2	63	1
80	12	3	65	1
80	13	1	66	1
80	13	2	65	1
80	14	2	64	1
80	15	1	64	1
80	16	1	63	1
80	16	2	62	2
80	17	1	62	1
81	13	3	65	1
81	14	2	65	4
81	15	2	64	2
82	13	2	67	5
82	14	2	66	1
82	14	3	65	1
82	15	2	65	2
82	18	1	63	2
83	12	3	68	1
83	13	2	68	1
83	15	2	66	2
83	19	2	62	1
84	14	2	68	1
85	15	2	68	1
86	15	0	71	1
87	18	1	68	1
87	19	1	67	1
90	17	3	70	1
98	14	2	82	1
Average 81.4	14.6	1.9	64.9	

*Metacentrics and submetacentrics.
†Subtelocentrics.
‡Telocentrics.

centric chromosomes, one having its second arm longer than that of the other. This is particularly well shown in figures 4 and 11. However, occasionally a cell may contain only 1 of these 2 chromosomes such as the one shown in figure 12.

The karyological characteristics for strain CF1-C-9 can be summarized briefly by the formula used in the previous section: M13-15:S2:T64-66 (80–82).

Chromosomes of Strain NCTC 2071, Clone 929 (Strain L)

NCTC clone 929 (strain L), subline NCTC 2071, isolated by Earle and his associates in chemically defined, protein-free medium (*12*), was also a heteroploid, with a low frequency (not more than 1%) for high polyploids. Figures 13 and 14 show the chromosomal characteristics of 2 cells of this strain, and figures 17 through 20 are ideograms of 4 different cells (telocentrics excluded). Table 3 summarizes the observational data on the chromosome types from 50 cells.

The average number of metacentric chromosomes per cell was 18, a value significantly higher than that of the 2 strains just reported. Among the metacentrics there were several long elements with unequal arms and 1 or 2 small chromosomes. There was also a conspicuous metacentric chromosome, which can be recognized in every cell. It was the longest element of the complement and its 2 arms were approximately equal in length. The most characteristic feature of this chromosome was that the 2 chromatids were always tightly held together even though the rest of the chromosomes were at the late stages of C-mitosis. In both photomicrographs (figs. 13 and 14) this chromosome can be seen. In the ideograms (figs. 17 through 20) it is placed as the first chromosome in each cell.

Several other cytological characteristics are also worth mentioning: 1) The average number of subtelocentrics for this line was approximately 4, a value also higher than that of the 2 previous bone-marrow cell strains. 2) Among the telocentrics there was an extraordinarily small element similar to the "minutes" reported in some mouse tumors. This chromosome was observed repeatedly and seemed to be one of the consistent members of the chromosome complex in this line. 3) Dicentric chromosomes were found in 5 cases out of nearly 1,000 cells inspected. Grossly, they were of 2 kinds. One type, recorded only once, was a giant chromosome approximately twice as long as the longest M chromosome of the set (fig. 16). The other 4 dicentrics appeared similar in length as well as in shape and were only slightly longer than the long M chromosomes. Since the present strain was established from a single cell, the dicentric element would have been in a fair proportion of the descendants if it were in the original cell. It is thus assumed that the dicentric chromosomes originated after the clone had been isolated. 4) A bizarre cell containing only 30 chromosomes was recorded (fig. 15). Judging from the completeness of the cell outline, the cell was probably the result of previous mitotic anomaly such as multipolar spindle.

In summary, this strain can be described as M17-19: S3-5: T50-52(72-74).

TABLE 3.—*Distribution of chromosome types in 50 cells of strain NCTC 2071, clone 929 (strain L)—high polyploids not included; chromosome fragments not recorded*

Number of chromosomes	M*	S†	T‡	Number of cells
69	16	4	49	1
70	17	3	50	1
71	15	3	53	1
71	15	4	52	1
71	17	3	51	1
71	18	3	50	1
71	19	3	49	1
71	19	4	48	3
71	20	4	47	1
72	21	4	47	1
72	17	4	51	1
72	18	2	52	1
72	18	4	50	3
72	19	3	50	1
72	19	4	49	2
73	16	5	52	1
73	17	4	52	1
73	18	3	52	1
73	19	4	50	1
74	16	4	54	2
74	17	3	54	3
74	17	4	53	2
74	17	5	52	1
74	18	3	53	1
74	18	4	52	5
74	19	4	51	1
74	20	3	51	1
74	21	2	51	1
75	14	6	55	1
75	15	4	56	1
75	16	4	55	1
75	18	4	53	2
75	19	4	52	1
76	16	4	56	1
76	17	5	54	1
76	18	4	54	1
Average 73.1	17.7	3.8	51.6	

*Metacentrics and submetacentrics.
†Subtelocentrics.
‡Telocentrics.

Chromosomes of Strain L-P55

In the fall of 1955, a piece of cartilage from a human fetal foot was cultivated in the laboratory of Dr. C. M. Pomerat. After a few transfers, a vigorous fibroblast cell line was established. It was designated as strain FF. Hsu and Moorhead (*1*) reported its chromosome number in 1957 and described it as heteropoloid. After its karyotype was studied in detail, it was discovered that the cytological characteristics of strain FF were more like those of murine origin than those of human origin because there were more telocentric elements.

Further evidence besides the cytological features was obtained later to prove that strain FF was from mouse tissue instead of man: 1) Inoculation of the cells into X-irradiated (425 r) C3H mice produced sarcoma-

tous tumors. One tumor was retransplanted to 9 nonirradiated C3H mice and 2 developed small tumors. 2) Inoculation of the cells into patients with malignant diseases failed to yield tumorous growth (personal communications with Dr. Alice E. Moore and Dr. Chester M. Southam, both of the Sloan-Kettering Institute, New York, who kindly performed the experiments for TCH). 3) Several other laboratories have had similar experience when cells from one established strain contaminated and replaced another culture (*13*). Personal communications with other investigators revealed that strain L of the mouse repeatedly contaminated other stocks. 4) The chromosomal characteristics of strain L_2 (supplied through courtesy of Dr. Sergey Fedoroff, Department of Anatomy, University of Saskatchewan, Saskatoon, Canada) resembled strikingly those of FF. Strain L_2 is a subline of clone 929 carried in Fedoroff's laboratory for a number of years and had no contact either with Pomerat's laboratory or our laboratory. But a conspicuous dicentric chromosome was present in both L_2 and FF, even though we have not proved that these 2 elements are identical. Probably, when workers in Pomerat's laboratory attempted to establish a cell strain from the fetal tissue, a single or a few L cells (clone 929 was also carried in that laboratory during that period) were accidentally introduced into a culture vessel containing the fetal tissue and later completely wiped out the human cells. Without cytological examination this error would not have been discovered and the wrong label would have been applied, thus confusing the literature. We redesignate the strain L-P55 as a substrain of clone 929 to replace the original symbol FF.

The most striking cytological feature of strain L-P55 was a long, dicentric chromosome. The majority of the cells contained either 1 or 2 such chromosomes that were apparently of a common origin, for all were alike in size as well as in shape. The element was the longest of the entire set, with 2 submedian constrictions. The longest arm was the middle piece, and 1 terminal piece was slightly longer than the other. Both constrictions of this chromosome were centromeric. In colchicine-treated material, when the chromatids are widely separated at late mitotic stages, the only place that holds the sister chromatids together is the centromeric constriction (not the secondary constriction, if it is present). In the chromosome in question, in strain L-P55, both constrictions held the chromatids. This is especially well shown in the ideograms, figures 26 through 30. Chu and Giles (*9*) also found several cells with dicentric chromosomes in the HeLa strain.

A small portion of the cells in this strain displayed no dicentric chromosome (fig. 21). Figure 22 shows a cell with 1 dicentric, and figure 23, a cell with 2 dicentrics. The frequent occurrence of chromatin bridges during anaphase may be due to this aberrant chromosome.

There were generally 13 metacentric and submetacentric chromosomes per cell. The lowest number of metacentrics registered was 9 and the highest, 16. In most of the cells there was an extraordinarily long metacentric chromosome that almost reached the length of the dicentric, and

an extraordinarily short element. The rest of the V's were of various lengths. The 2 arms of most of the metacentrics were approximately equal in length, but a few were unequal. There were about 4 (range 2 to 6) subtelocentrics in each cell. Most of them were relatively short, but occasionally a longer one was observed. The number of the telocentrics varied from 45 to 55.

Table 4 summarizes the recording data on 50 cells of this strain with respect to their chromosome types. Four complete ideograms (figs. 25 through 28) are also presented to illustrate the heterogeneity of the

TABLE 4.—*Distribution of chromosome types in 50 cells of strain L-P55—high polyploids not included; chromosome fragments not recorded*

Number of chromosomes	M*	S†	D‡	T§	Number of cells
63	13	2	2	46	1
63	13	4	1	45	1
65	12	4	1	48	1
65	13	4	1	47	1
65	14	4	1	46	1
65	15	3	1	46	1
66	11	3	2	50	1
66	12	5	1	48	1
66	14	3	0	49	1
66	14	3	2	47	1
67	9	4	2	52	1
67	12	2	2	51	1
67	12	3	2	50	1
67	13	2	2	50	1
67	13	5	1	48	1
67	14	4	0	49	1
67	15	3	1	48	1
67	15	4	1	47	1
67	16	4	1	46	1
68	12	4	1	51	1
68	13	3	2	50	1
68	13	4	1	50	2
68	13	5	1	49	1
68	13	6	0	49	1
68	14	5	1	48	1
69	9	5	2	53	1
69	11	4	2	52	1
69	12	3	2	52	1
69	12	4	1	52	1
69	13	3	0	53	1
69	13	3	2	51	2
69	13	4	1	51	5
69	13	4	2	50	1
69	14	4	1	50	1
69	15	5	0	49	1
70	11	3	1	55	1
70	12	5	1	52	1
70	13	3	1	53	2
70	13	4	1	52	1
70	13	4	2	51	1
70	13	5	1	51	1
70	14	3	1	52	1
70	14	4	1	51	1
Average 67.9	12.9	3.8	1.2	49.9	

*Metacentrics and submetacentrics.
†Subtelocentrics.
‡Dicentrics.
§Telocentrics.

genetic background of this strain. Figure 25 was chosen for its absence of the dicentric chromosome, figure 26 for the presence of 1 dicentric, and figures 27 and 28 for having 2 such elements. Other chromosomal features were drawn as they happened to be.

Like the cell lines described in the previous sections, practically every cell in strain L-P55 under critical examination revealed in one way or another some karyological characteristics of its own. For the sake of simplicity, however, the chromosomal features of this strain can be described with this simple formula: D1-2: M12-14: S4: T49-51(67-70).

Chromosomes of Strain L-P55-N2

Strain L-P55-N2 is a substrain of L-P55 and was established in the spring of 1957—again by accidentally introducing a single cell or a few cells to a cell line of rat hepatoma. Karyotypically, the chromosomes of this substrain were very similar under casual observation to those of L-P55. Both possessed the peculiar dicentric element and had over 10 metacentrics and subtelocentrics and many telocentrics. After more critical examination, however, a number of subtle cytological differences were noted between these 2 lines. Table 5 lists 50 cells analyzed for the distribution of chromosome types of strain L-P55-N2, subculture 26.

The following points are the major cytological characteristics that could differentiate L-P55-N2 from its mother strain L-P55: 1) Every cell in L-P55-N2 contained either 1 or 2 dicentrics, whereas in strain L-P55 a small fraction of the population was void of such an element. 2) Substrain L-P55-N2 had a more constant karyotype than its mother strain. If the variations in total chromosome number and in the number of the dicentrics were disregarded, the strain was characterized by having 12 metacentrics and 2 subterminals. 3) Among the metacentrics, the extremely long chromosomes with equal arms that occurred frequently in the cells of L-P55 were absent. Instead, in the populations of L-P55-N2 there was a submetacentric chromosome that slightly exceeded the dicentric in length. Besides, this chromosome was found in every cell studied. 5) One of the 2 subtelocentrics was much longer than the other, nearly reaching the length of the middle-sized metacentrics. This chromosome was found in L-P55-N2 in almost every cell.

This substrain L-P55-N2, therefore, can easily be described by a simple formula: D1-2:M12:S2:T52(67-68). Three photomicrographs and 2 ideograms illustrate the characteristic points. Two of the photomicrographs (figs. 22 and 23) have been mentioned before. In figure 24, besides the dicentric, there is a configuration suggesting a new structural rearrangement. Figures 29 and 30 are complete ideograms, one showing a cell with a single dicentric (fig. 29) and the other, 2 dicentrics (fig. 30). Both had 12 metacentrics (note the longest submetacentric) and 2 subtelocentrics (note the longer element).

Substrain L-P55-N2, with its own special cytological features, was thus more homogeneous in chromosomal constitution than its mother strain L-P55.

TABLE 5.—*Distribution of chromosome types in 50 cells of strain L-P55-N2—high polyploids not included; chromosome fragments not recorded*

Number of chromosomes	M*	S†	D‡	T§	Number of cells
64	13	2	1	48	2
64	14	3	1	46	1
65	13	2	1	49	1
66	12	2	1	51	2
66	12	2	2	50	1
67	12	2	1	52	4
67	12	2	2	51	4
67	12	3	1	51	1
67	13	1	1	52	1
67	13	2	1	51	1
67	13	2	2	50	1
67	14	2	2	49	1
68	11	3	2	52	1
68	12	1	2	53	1
68	12	2	1	53	2
68	12	2	2	52	7
68	13	2	1	52	1
68	13	2	2	51	2
68	14	2	2	50	1
68	16	2	2	48	1
69	11	2	2	54	1
69	12	2	2	53	6
69	13	2	1	53	1
69	13	2	2	52	1
70	11	3	2	54	1
70	12	2	2	54	1
71	14	3	1	53	1
72	16	2	1	53	1
75	12	2	1	60	1
Average 67. 9	12. 5	2. 0	1. 6	51. 7	

*Metacentrics and submetacentrics.
†Subtelocentrics.
‡Dicentrics.
§Telocentrics.

Comparison of the Chromosomal Contents among Populations

In the preceding sections, tables 1 to 5 enumerated, cell for cell, the observed chromosome structures. Presentation of raw data enables anyone to derive his own method of statistical treatment. However, in order to make a comparison of the chromosomal contents among the populations and to detect whether there is any correlation between the number of the biarmed and multiarmed chromosomes and that of the total chromosomal elements, it is relatively difficult to grasp the significance by inspecting the original tables.

It is true that a metacentric chromosome may arise from a fusion between 2 telocentrics or from a pericentric inversion within a telocentric chromosome. In normal mouse cells, all the 40 chromosomes are telocentric. Just how many metacentrics in the cell strains derived their origin by fusion or translocation and how many by pericentric inversion cannot be ascertained; but judging by the enormous size of most of the metacentric and subtelocentric elements, we can reasonably assume that a

greater portion of them came into being by fusion. It is even more difficult to determine the origin of the dicentric. There is no doubt that a translocation has taken place, but the chromosome may represent either 3 or 4 original telocentric segments.

For the sake of expediency in comparing the cytological contents of the cell lines, we shall study the populations with respect to the chromosome arms, instead of the chromosomes, and consider the dicentric as being composed of 4 arms. A regrouping of the data presented in tables 1 to 5 yields tables 6 to 10. These tables provide a bird's-eye view of the

TABLE 6.—*Correlation between the total number of chromosome arms and the number of biarmed chromosomes in strain MC3*

Combinations of M and S chromosomes																
M		12	13	14			15		16			17		18	19	Total
S		2	2	1	2	3	1	2	0	1	2	1	2	1	3	
Number of chromosome arms	91						1									1
	92						1	1	1	1						4
	93	1	1		1											3
	94			1	1		1				1					4
	95			1	1		3	2		6	1	1	1			16
	96		1				2			3		2				8
	97			1						2	2	1		1		7
	98										2					2
	99					1					1		1			3
	100											1			1	2
Total.....		1	2	3	3	1	8	3	1	12	7	5	2	1	1	50

TABLE 7.—*Correlation between the total number of chromosome arms and the number of biarmed chromosomes in strain CF1-C-9*

Combinations of M and S chromosomes																		
M		12	13			14		15			16		17		18	19		Total
S		3	1	2	3	2	3	0	1	2	1	2	1	3	1	1	2	
Number of chromosome arms	83			1														1
	92			1														1
	93		1	1					1	1								4
	94		1															1
	95	1		1		3				1								6
	96					1			1									2
	97			5	1	4				1	1							12
	98	1		1		1				2		2	1					8
	99						1			2								3
	100					1				2								3
	101							1						2				3
	102									1								1
	104																1	1
	106													1				1
	107														1			1
	110												1					1
	114					1												1
Total.....		2	2	10	1	11	1	1	2	10	1	2	1	1	3	1	1	50

relationship between the total number of chromosome arms per cell and the number of the biarmed and multiarmed chromosomes. In all the tables the distribution of the recorded values seems to run diagonally from the upper-left to the lower-right corner, an indication of some degree of positive correlation between the 2 factors. This suggests that the variation of the number of the biarmed and multiarmed chromosomes contributes to the variation of the total number of chromosome arms. In table 10, however, there is a slight tendency for condensation of the distributional values on certain vertical columns, suggesting that the variation of chromosomal material in this population was somewhat a function of the variation of the telocentrics.

TABLE 8.—*Correlation between the total number of chromosome arms and the number of biarmed chromosomes in strain NCTC 2701, clone 929 (strain L)*

	Combinations of M and S chromosomes																
M	14	15		16		17			18			19		20		21		Total
S	6	3	4	4	5	3	4	5	2	3	4	3	4	3	4	2	4	
Number of chromosome arms 89		1		1														2
90			1			1												2
91						1												1
92									1	1								2
93							1					1						2
94			1	2	1	3	1			1	3	1	3					16
95	1			1			2			1			2		1			8
96				1				1			5		1					8
97											2		1	1		1	1	6
98								1			1		1					3
Total...	1	1	2	5	1	5	4	2	1	3	11	2	8	1	1	1	1	50

When the number of the chromosome arms in the populations is considered, the first 3 strains are more similar (all in the middle 90's) than the last 2 (in the upper 80's) that contained the dicentric element. However, if we assume that each arm represents a telocentric chromosome and disregard the possible reshuffling of the genetic material, all the cell lines can be placed in the category of hypertetraploid.

Discussion

It is rather astonishing to learn that in cell strains established originally from normal tissues not only the chromosome number is changed from diploid to heteroploid but also the chromosome alignment is altered drastically. From the 40 telocentric chromosomes in the diploid mouse cell it would take numerous chromosomal breakages to reach the karyotypes in the cell strains described. The 10 to 20 metacentrics and subtelocentrics are entirely new to the normal mouse cells, not including the giant dicentric element. Similar procedures probably take place in human cell strains since Chu and Giles (*9*) found telocentric elements in the HeLa clones, whereas in normal human karyotype each chromosome has 2 arms. Reciprocal translocation between 2 chromosomes of the

TABLE 9.—*Correlation between the total number of chromosome arms and the number of biarmed and multiarmed chromosomes in strain L-P55; each dicentric chromosome is considered as 4 arms*

	Combinations of M, S, and D chromosomes																															Total
M	9		11			12				13								14						15			16					
S	4	5	3		4	2	3	4	5	2	3			4			5	6	3			4		5	3			4				
D	2	2	1	2	2	2	2	1	1	2	0	1	2	1	2	1	0	0	1	2	1	0	1	1	1	0	1					
Number of chromosome arms 83														1				1														2
84								1		1																						2
85											1			1									1									3
86	1			1					1												1			1							5
87			1			1		1									1															4
88							1	1		1				2		1								1								7
89		1										2		5	1	1				1					1	1						13
90					1		1		1				1	1					1		1		1				1					9
91													2			1					1											4
92															1																	1
Total...	1	1	1	1	1	1	2	3	2	2	1	2	3	10	2	3	1	1	1	1	3	1	1	2	1	1	1					50

TABLE 10.—*Correlation between the total number of chromosome arms and the number of biarmed and multiarmed chromosomes in strain L-P55-N2; each dicentric chromosome is considered as 4 arms*

Combinations of M, S, and D chromosomes															Total
M		11		12				13			14		16		
S		2	3	1	2		3	1	2		2	3	2		
D		2	2	2	1	2	1	1	1	2	2	1	1	2	
Number of chromosome arms	82								2						2
	83				2				1						3
	84				4			1				1			6
	85				2		1		1						4
	86					1			1						2
	87			1		4			1						6
	88	1	1			7				1					10
	89					6				2	1				9
	90		1			1				1	1				4
	91											1			1
	92				1									1	2
	93												1		1
Total....		1	2	1	9	19	1	1	6	4	2	2	1	1	50

same shape might occur more often, but most of them would escape detection even after careful ideogrammatic analyses. Even in neoplasms *in situ*, similar profound alterations of chromosomal structure have been found (*14*). A careful analysis made by Tjio and Levan (*15*) on the chromosomes of the Yoshida sarcoma revealed that the tumor also had undergone numerous structural interchanges from the normal rat karyotype to its present status. Had the Yoshida sarcoma not had the conspicuous metacentric chromosome and the reduction of the diploid number, its minor but more numerous alterations of genic alignments might not have been discovered. Cancer cytologists would have to keep in mind that many diploid mouse tumors may also contain a number of such alterations of chromosome structure.

The process of chromosomal rearrangement, in addition to the process of heteroploid transformation in cell strains, has been witnessed in our strains of the Novikoff rat hepatoma (unpublished data). Thus, the transformation actually brings about a profound reshuffling of the genetic material rather than a mere change of ploidy. Perhaps it would be rather misleading, though expedient, to designate any cell strains as triploid, hypotetraploid, 3x—4, 4x—3, etc., for such simple combinations do not in reality exist in cell lines.

From the karyograms of the strains it is highly suggestive that during the stages of transformation certain heteroploid cells with structural rearrangements seem to possess metabolic superiority over their competitors and give rise to the cell line. When an *in vitro* cell strain is established from biopsy or autopsy material, it is generally presumed, again due to the lack of criteria to differentiate individual cellular elements, that the strain is the progeny of a number of cells which adapted to the new environment. It appears reasonable to suppose that each of these cells produces a colony that mixes with others to produce the cell line. Nevertheless, it is also conceivable that one colony may be metabolically superior to others in this challenging environment, thus gradually dominating the population. In the present case this hypothesis seems to be applicable. Each strain had its chromosomal characteristics as though the cells were the descendants of a single cell.

Since the probability of a particular structural rearrangement occurring twice at precisely the same loci is mathematically negligible, it is correspondingly safe to conclude that cells possessing the long dicentric chromosome in strains L-P55, L-P55-N2, and probably L_2 (Fedoroff) are the descendants of a single mother cell. Those without the dicentric in L-P55 were probably also from the same ancestral cell since the pattern of other chromosomes resembled that in the cells with the dicentric. Two possibilities exist: 1) The dicentric translocation took place after the rest of the changes had been completed. 2) All the cells originally had the dicentric, but a part of them lost this peculiar chromosome due to its misbehavior during mitotic division. The former possibility seems to be more plausible because none of the cells in substrain L-P55-N2 lost this element after 6 months of continuous cultivation.

An interesting question arises from the foregoing discussion: What would be the fate of a cell losing its dicentric chromosome? Would it be unable to continue its struggle for survival because of the loss of some vital enzymes or enzyme makers? Or would the dicentric always divide equally like the rest of the chromosomes? We have observed a number of cells with 2 dicentrics, which would indicate abnormal mitosis in the previous cell generations and that the dicentric did not divide equally at all times. The fact that the dicentric always retained its characteristic length and arm proportions also suggested that breakage and reunion of the bridge did not occur frequently or, whenever it happened, the daughter cells were not able to compete with the "standard" types and were thus eliminated from the population. On the other hand, when there was a chance for cells to acquire 2 such dicentrics, there should be a chance for them to acquire 3. The fact that all the cells of L-P55-N2 (26 subcultures after its establishment) possessed at least 1 but not more than 2 dicentrics indicates that the genic material in this chromosome was essential but could not be overdosed. As has been suggested previously, the translocation process that caused the formation of the dicentric was probably of later occurrence. Even though cells containing this chromosome were superior, cells having the complement prior to the dicentric change were

still able to survive. It is possible, however, that cells lacking the dicentric will gradually be eliminated from mixed populations, though they may well maintain themselves in clone cultures.

The phenomenon of genetic polymorphism in the strains studied in this report indicates that a great many cell strains carried in various laboratories are not homogeneous. Our unpublished data on the chromosomes of the Novikoff hepatoma, especially when the cells were in the stages of heteroploid transformation, provide some extremely exaggerated cases of cytological polymorphism. In a mixed population various genotypes, unless they are grossly deficient or unsuitable for struggle, probably benefit each other symbiotically, giving away certain metabolites that they are able to manufacture in surplus while receiving some from other genotypes. It is reasonable to assume that all the existing cells have some doses of essential genetic material. Under isolated conditions such as in the clonal strains, however, some cells may not perform their metabolic activities as efficiently as others. The difference in "plating efficiency" between the clones S1 and S3 of HeLa may well be explained by this reasoning.

Since the cells in tissue-culture strains have lost their tissue organization and become somewhat individualized, the environment in a culture vessel for the cells is perhaps analogous to the natural environment for individual organisms of a species. In nature, genetic polymorphism is not only a common feature but probably a "must" for a species to survive through numerous harsh selective forces that act upon it (*16*). Here in cell strains, heterogeneity of chromosomal constitution may also be beneficial to the population as a whole. It has been emphasized (*6*, *17*) that in a given environment and at a given time one genotype may dominate the population to become the so-called "stem line," while others, in a minority, serve as reserves to deal with adverse environmental changes. Thus, one type of cells may become the stem line at one time but the population is never fixed. In other words, stem line is a temporary cell type liable to be replaced by another genotype that may become the new stem line. In their report on the sublines of the Yoshida sarcoma, Makino and Sasaki (*18*) also modified their original stemline concept to one which is consistent with our views here.

It is important to remember that contamination of one cell strain by another falsifies the observational and experimental data. It has been well documented that cells in many tissue-culture strains are morphologically indistinguishable (*19*), regardless of their origin. As previously mentioned, contamination of one species by another has happened in several laboratories. At least in 3 cases that we are aware of (1 reported here) the catastrophe was averted by cytological identification. Not all the cell strains, however, have detailed cytological data, and presumably not all the strains are karyologically identifiable. In the present case, our laboratory received the strain FF from Dr. Pomerat's laboratory, and we sent the strain with the same label to Dr. Moore who was puzzled that such a supposedly malignant line failed to produce any tumorous

growth. Had Dr. Levan not informed us that the chromosomes of strain FF appeared very much like some mouse cell lines he had seen before, we would not have traced its origin through a number of possible avenues of identification. Indeed, there is a strong possibility that strains MC3 and CF1-C-9 may also be the results of L-strain contamination. Recently, Drs. K. Rothfels, L. Siminovitch, and A. Axelrad (personal communication) demonstrated immunologically as well as cytologically that these lines are actually L strains. It is highly desirable for every laboratory to set up a very rigid system of stock maintenance, especially when vigorous cell lines such as HeLa and L are being carried.

References

(1) Hsu, T. C., and Moorhead, P. S.: Mammalian chromosomes *in vitro*. VII. Heteroploidy in human cell strains. J. Nat. Cancer Inst. 18: 463–471, 1957.

(2) Berman, L., Stulberg, C. S., and Ruddle, F. H.: Human cell culture. Morphology of the Detroit strains. Cancer Res. 17: 668–676, 1957.

(3) Levan, A.: Chromosome studies on some human tumors and tissues of normal origin, grown *in vivo* and *in vitro* at the Sloan-Kettering Institute. Cancer 9: 648–663, 1956.

(4) Westwood, J. C. N., and Titmuss, D. H. J.: Transformation in tissue culture cell lines. The possible genetic mechanism. Brit. J. Exper. Path. In press.

(5) Hsu, T. C., Pomerat, C. M., and Moorhead, P. S.: Mammalian chromosomes *in vitro*. VIII. Heteroploid transformation in the human cell strain Mayes. J. Nat. Cancer Inst. 19: 867–873, 1957.

(6) Hsu, T. C.: Numerical chromosomal variations in higher animals. *In* Developmental Cytology (Rudnick, D., ed.). New York, Ronald Press Co. In press.

(7) Hansen-Melander, E., Kullander, S., and Melander, Y.: Chromosome analysis of a human ovarian cystocarcinoma in the ascites form. J. Nat. Cancer Inst. 16: 1067–1081, 1956.

(8) Puck, T. T., and Fisher, H. W.: Genetics of somatic mammalian cells. I. Demonstration of the existence of mutants with different growth requirements in a human cancer cell strain (HeLa). J. Exper. Med. 104: 427–434, 1956.

(9) Chu, E. H. Y., and Giles, N. H.: Comparative chromosomal studies on mammalian cells in culture. I. The HeLa strain and its mutant clonal derivatives. J. Nat. Cancer Inst. 20: 383–401, 1958.

(10) McCulloch, E. A., and Parker, R. C.: Continuous cultivation of cells of hemic origin. Canadian Cancer Conf. 2: 152–167, 1957.

(11) Ford, C. E., and Hamerton, J. L.: A colchicine, hypotonic citrate, squash sequence for mammalian chromosomes. Stain Technol. 31: 247–251, 1956.

(12) McQuilkin, W. T., Evans, V. J., and Earle, W. R.: The adaptation of additional lines of NCTC clone 929 (strain L) cells to chemically defined protein-free medium NCTC 109. J. Nat. Cancer Inst. 19: 885–907, 1957.

(13) Ford, D. K., and Yerganian, G.: Observations on the chromosomes of Chinese hamster cells grown in tissue culture. J. Nat. Cancer Inst. 21: 393–425, 1958.

(14) Ising, U., and Levan, A.: The chromosomes of two highly malignant human tumors. Acta path. et microbiol. scandinav. 40: 13–24, 1957.

(15) Tjio, J. H., and Levan, A.: Comparative ideogram analysis of the rat and the Yoshida rat sarcoma. Hereditas 42: 218–234, 1956.

(16) Dobzhansky, T. G.: Genetics and the Origin of Species. New York, Columbia Univ. Press, 1951, 374 pp.

(17) Hsu, T. C.: Lecture at Gordon Cancer Research Conference. New London, N. H., 1957.

468155—58——3

(*18*) MAKINO, S., and SASAKI, M.: Cytological studies of tumors. XXI. A comparative ideogram study of the Yoshida sarcoma and its subline derivatives. J. Nat. Cancer Inst. 20: 465–488, 1958.

(*19*) LEIGHTON, J., KLINE, I., BELKIN, M., LEGALLAIS, F., and ORR, H. C.: The similarity in histologic appearance of some human "cancer" and "normal" cell strains in sponge-matrix tissue culture. Cancer Res. 17: 359–363, 1957.

PLATE 53

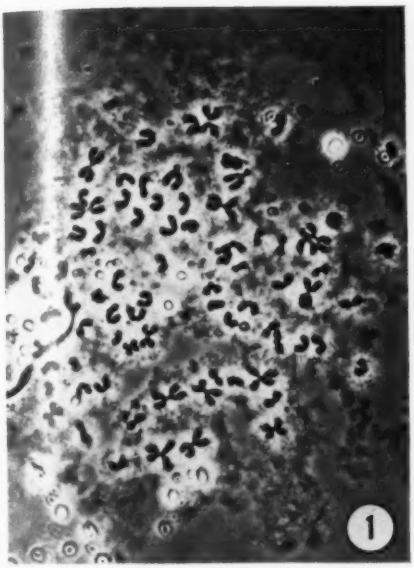
Photomicrographs of cells from strains MC3 and CF1-C-9. Squash preparations, pretreated with colchicine and hypotonic solution. No stain, phase-contrast microscopy. For magnification *see* scale on figure 4.

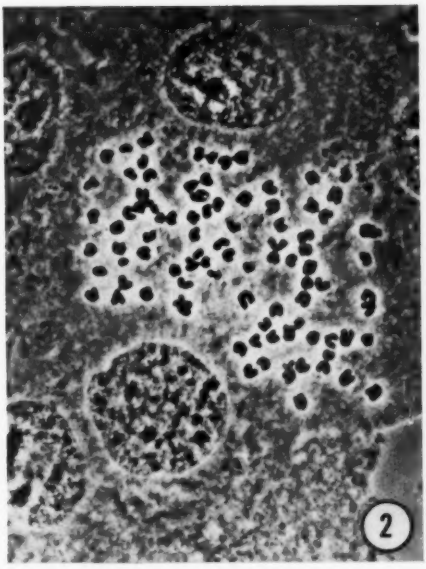
FIGURE 1.—A cell from strain MC3. *Note* the 2 seemingly identical subtelocentric chromosomes at *top* of figure.

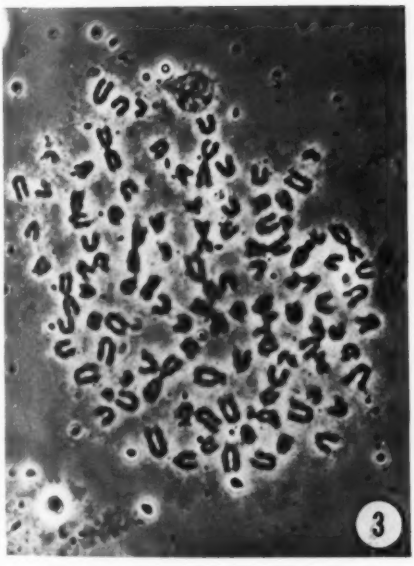
FIGURE 2.—A cell from strain MC3. *Note* the single subtelocentric chromosome at 3 o'clock.

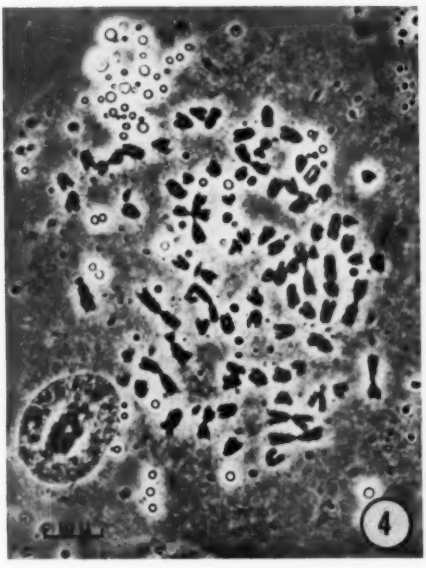
FIGURE 3.—A cell from strain CF1-C-9. *Note* the 2 different subtelocentric chromosomes, 1 at the *lower center* of picture and the other near 5 o'clock.

FIGURE 4.—A cell from strain CF1-C-9. *Note* the 2 different subtelocentric chromosomes, 1 at 9 o'clock and the other near 8 o'clock.







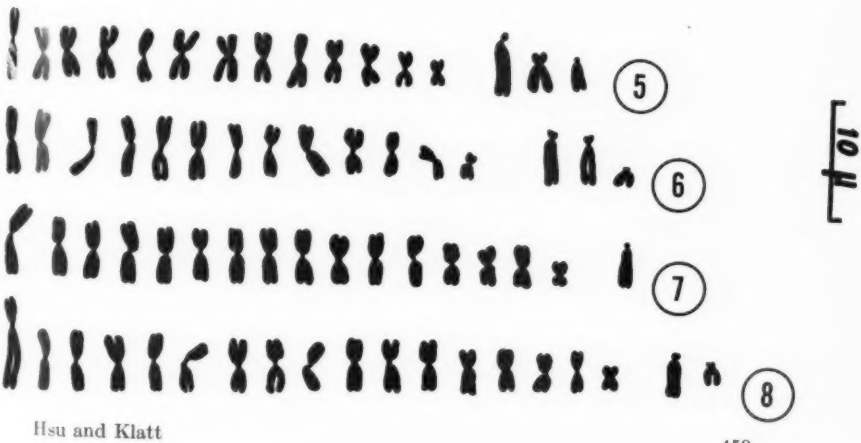


Hsu and Klatt

468155—58——4

PLATE 54

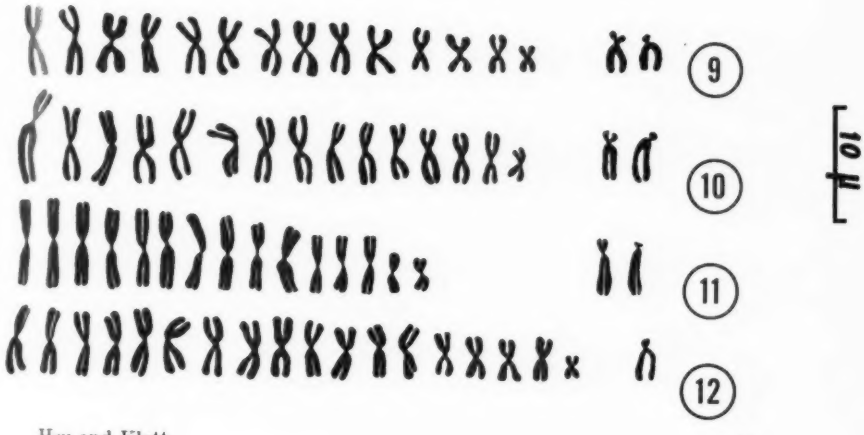
FIGURES 5 to 8.—Ideograms of 4 cells of strain MC3 showing variation in karyotype. Telocentric chromosomes not drawn.



Hsu and Klatt

PLATE 55

FIGURES 9 to 12.—Ideograms of 4 cells of strain CF1-C-9 showing variation in karyotype. Telocentric chromosomes not drawn. *Note* the 2 different subtelocentric chromosomes in figures 9 to 11. In figure 12 only 1 can be found.



Hsu and Klatt

PLATE 56

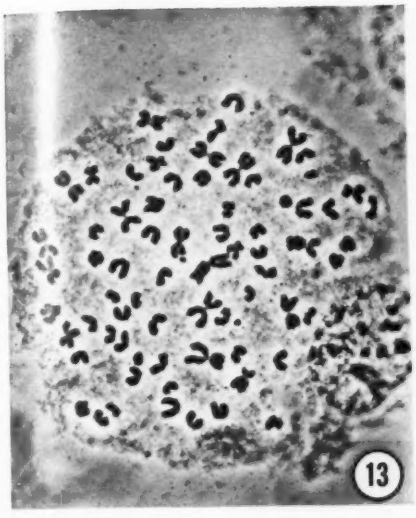
Photomicrographs of cells from strain NCTC 2071, clone 929 (strain L). Squash preparations, pretreated with colchicine and hypotonic solution. No stain, phase-contrast microscopy. For magnification *see* scale on figure 4.

FIGURE 13.—A cell with 73 chromosomes and a fragment. *Note* at *center* of plate the long metacentric chromosome that has its chromatids held close together. *Also note* the minute telocentric element at 2 o'clock.

FIGURE 14.—A cell with 75 chromosomes and a fragment. *Note* the long metacentric chromosome at 4 o'clock and the minute telocentric immediately below it.

FIGURE 15.—A cell with only 30 chromosomes. An unusual cell seen only once among nearly 1,000 cells inspected.

FIGURE 16.—A cell with a giant dicentric chromosome. The long metacentric chromosome can also be seen.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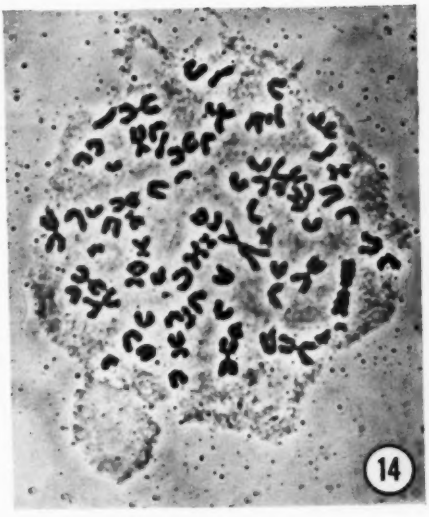
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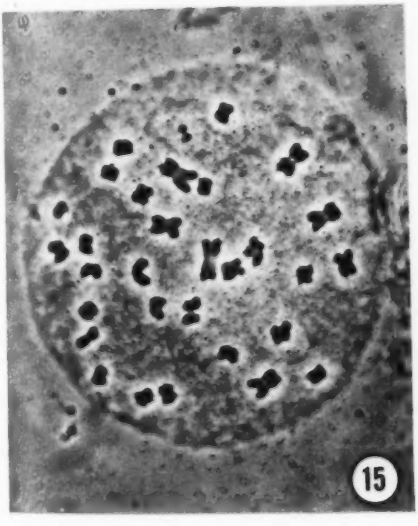

15

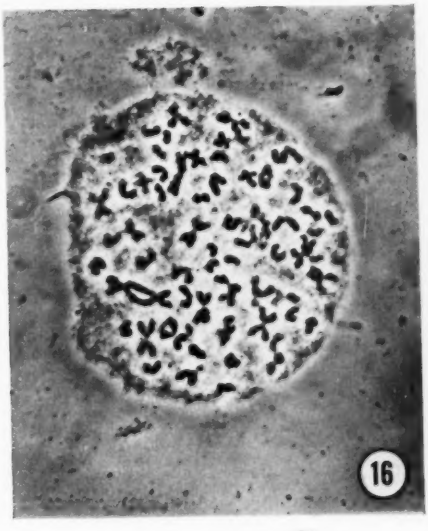

16

PLATE 57

FIGURES 17 to 20.—Ideograms of 4 cells of strain NCTC 2071, clone 929 (strain L), showing variation in karyotype. Telocentric chromosomes not drawn. *Note* the morphology of the long metacentric chromosome placed first in each row.

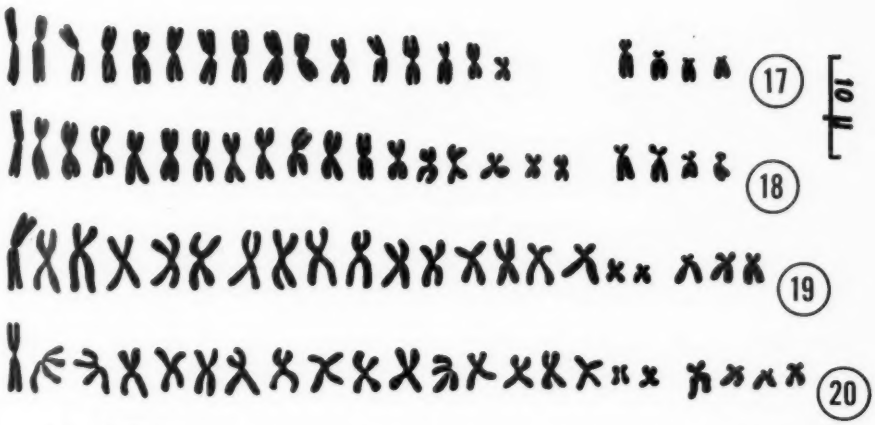

17
18
19
20
10 μ

PLATE 58

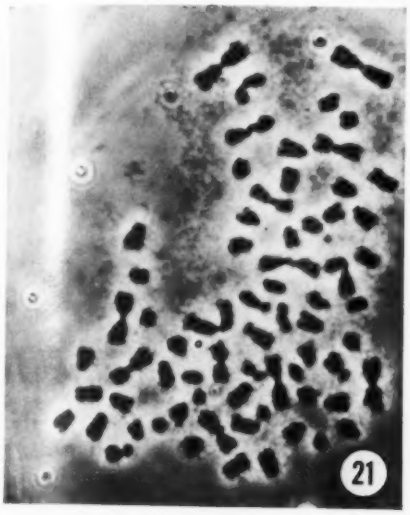
Photomicrographs of cells from strains L-P55 and L-P55-N2. Squash preparations, pretreated with colchicine and hypotonic solution. No stain, phase-contrast microscopy. For magnification *see* scale on figure 24.

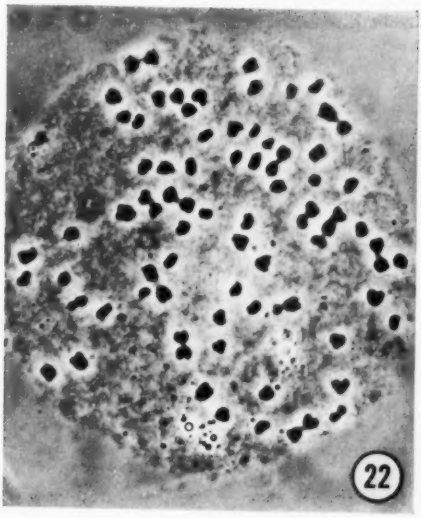
FIGURE 21.—A cell from strain L-P55 showing no dicentric chromosome.

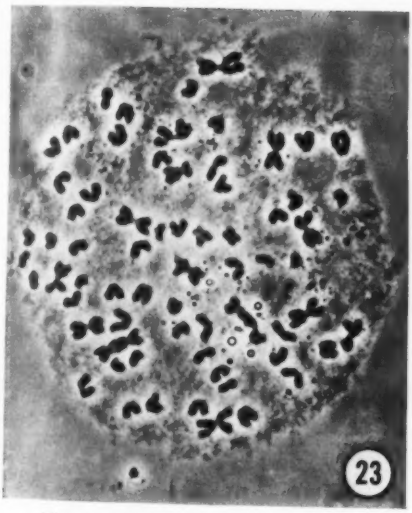
FIGURE 22.—A cell from strain L-P55-N2. *Note* the single dicentric chromosome near 3 o'clock.

FIGURE 23.—A cell from strain L-P55-N2. *Note* 2 dicentric chromosomes.

FIGURE 24.—A cell from strain L-P55-N2. *Note* the single dicentric chromosome and another configuration suggestive of translocation.


21


22


23

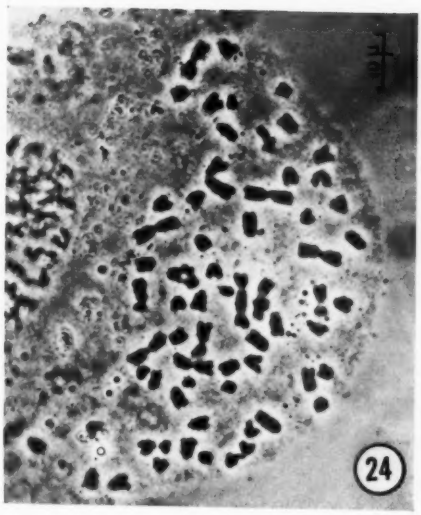

24

PLATE 59

Complete ideograms of 2 cells from strain L-P55.

FIGURE 25.—A cell with no dicentric chromosome. *Note also* presence of 2 extraordinarily long metacentric chromosomes.

FIGURE 26.—A cell with a single dicentric chromosome. *Note* presence of a single extraordinarily long metacentric chromosome.

25
26
10 μ

PLATE 60

FIGURES 27 and 28.—Complete ideograms of 2 cells from strain L-P55. Both cells contain 2 dicentric chromosomes and 1 extraordinarily long metacentric chromosome.

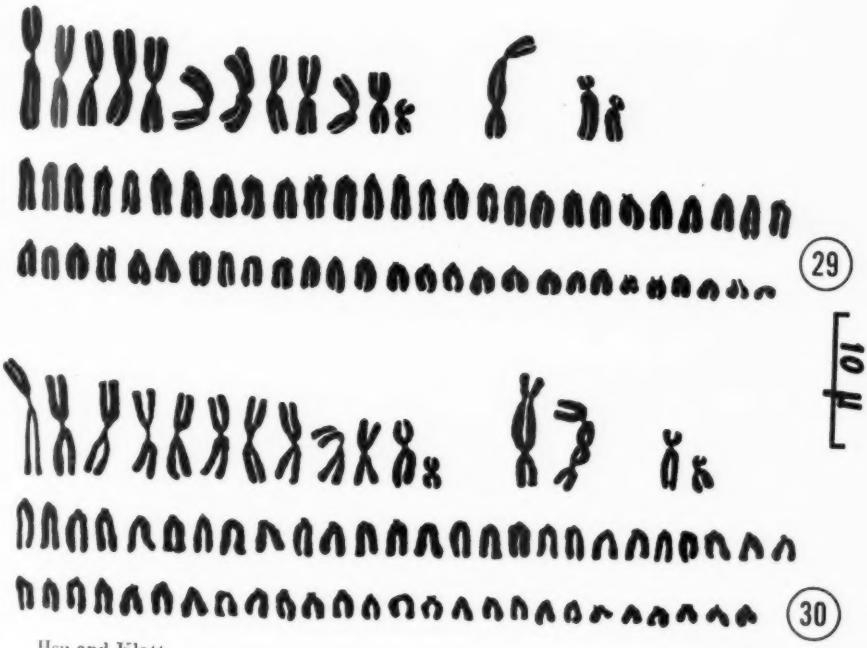
27
28
10 μ

PLATE 61

Complete ideograms of 2 cells from strain L-P55-N2. *Note* the extraordinarily long submetacentric chromosome and the 2 different subtelocentric chromosomes in each case. These characteristics can be used to differentiate this strain from its mother strain L-P55 (*cf.* figs. 25 to 28).

FIGURE 29.—A cell with 1 dicentric chromosome.

FIGURE 30.—A cell with 2 dicentric chromosomes.


29
10 μ
30

The Effect of Glucose Analogues on the Metabolism of Human Leukemic Cells [1]

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Summary

Certain sugar analogues (2-deoxy-D-glucose and 2-deoxy-D-galactose) were shown to be potent glycolytic inhibitors of human leukocytes, human leukemic cells of different types, and a variety of animal tumor cells. *In vivo* administration of 2-deoxy-D-glucose markedly inhibited glycolysis in 2 of 4 patients with leukemia studied. The possible usefulness of a technique measuring metabolic changes induced by a drug *in vivo* was discussed. **J. Nat. Cancer Inst. 21: 475–483, 1958.**

Data on the metabolism of human leukemic cells and the effects of chemotherapeutic agents on the cells have recently been reported from this laboratory (*1–8*). Antileukemic drugs (Methotrexate,[3] 6-mercaptopurine, and hydrocortisone) have been found to affect the glycolysis and respiration of white blood cells taken from selected patients with acute leukemia. Leukemic cells have been studied as prototypes of human neoplastic cells. The present investigations have been carried out to determine the effects of various glucose analogues on the metabolism of leukemic cells.

It has been previously reported by others (*9, 10*) that 2-deoxy-D-glucose (2-DG) inhibited glycolysis in brain, diaphragm, liver slices, and yeast cells. Glycolytic inhibition by 2-DG of Walker carcinosarcoma 256 and of Flexner-Jobling carcinoma has also been described (*9*). Growth inhibition of yeast cells and chick-heart fibroblasts has been observed (*10, 11*) which could be competitively overcome by glucose. Increase in survival time of tumor-bearing animals and decrease in tumor size has been noted after treatment with 2-DG in Krebs-2 ascites carcinoma (*6*), Walker carcinosarcoma 256 (*13*), Crocker rat carcinoma (*14*), and recently in ad-

[1] Received for publication March 20, 1958.

[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

[3] Amethopterin.

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vanced mouse Lymphoid leukemia L1210 (*15*). Since the original description of 2-DG competitive antagonism of glucose, Wick (*16*) has reported that the site of block under his experimental conditions appeared to be at the phosphohexoisomerase reaction. However, it is unlikely that this block by 2-deoxy-D-glucose-6-phosphate of the isomerase enzyme isolated from rat kidney is applicable to the cancer cell. Indeed, fructolysis is inhibited in the cancer cell more than glycolysis, and this process not only bypasses the isomerase step but would be expected on the basis of hexokinase inhibition considering the relative affinities of the 2 sugars for the enzyme site.

This report deals with data on glycolytic inhibition by 2-DG of various mouse tumors and human leukemic cells *in vitro* as well as glycolytic inhibition of leukemic cells following *in vivo* administration of 2-DG to patients with leukemia.

Materials and Methods

Sugar analogues.—2-Deoxy-D-glucose was purchased from the Aldrich Chemical Company. 2-Deoxy-D-galactose (2-D-Gal) was prepared by the method of Overend (*17*), 3-*O*-methyl-D-glucose and 3-*O*-methyl-D-fructose by the methods of Glen (*18*), 6-*O*-methyl-D-glucose by the method of Bell (*19*). 3-Deoxy-D-glucose (3-DG) was prepared by Dr. James Pratt and 6-deoxy-6-fluoro-D-glucose by Dr. Isadore Zipkin.

Human leukemic cells.—These were obtained from patients at the National Cancer Institute. Patients were selected for study if their white blood counts were in excess of 15,000 cells per mm^3, and if the predominant cell type was in the leukemic series. Eight leukemic patients were studied on one or more occasions. Included in the study were patients with acute and chronic lymphocytic leukemia, acute and chronic myelogenous leukemia, and acute monocytic leukemia. One nonleukemic patient with benign leukocytosis was also investigated.

Separation of human leukocytes for manometric studies by the fibrinogen-sedimentation method has been previously described (*2*). The white blood cells in autologous plasma were pipetted directly into manometric vessels. Glucose, 2.0 mg., and sodium bicarbonate, 1.5 mg. were added per ml. of fluid in Warburg manometric vessels. The sugar analogues were usually added to the vessels in powder form and were readily dissolved by the plasma. The compounds were added in concentrations varying from 0.5 to 10 mg. per ml. but usually 2.5 to 5.0 mg. per ml.

2-DG in vivo.—Administration *in vivo* was carried out by infusing 50 to 68 mg. per kg. 2-DG, during 30 to 60 minutes, to patients with leukemia and high white-blood-cell counts. Blood samples were drawn through a Cournand needle in the opposite arm before drug administration and at intervals thereafter. White cell suspensions in serum were prepared and manometric analysis performed in triplicate as described. Such experiments were also carried out by using citrated whole blood. Care was taken to assure that the elapsed time between the blood drawing and placing it on the water bath was approximately the same for each

sample. Cell counts were made on each sample and the results calculated as mm. gas produced per 10 million leukocytes per hour. Glucose determinations were made on samples before and after incubation in the manometers, and glucose utilization was calculated on the same basis. 2-DG blood levels were determined, when each sample was taken, by the quinaldine reagent method (*20*). Lactic acid was measured in certain experiments by the Barker-Summerson method (*21*). Due to the profound effect of 2-DG in raising blood glucose in fasting patients (*22*), one experiment was performed on patient C. F. in which glucose was added in graded quantities to the initial samples in an effort to approximate the incubated samples in regard to initial glucose concentrations.

Mouse tumor cells.—In several experiments the glucose analogues were incubated with the following mouse tumor cells: Krebs-2 ascites, Lymphocytic neoplasm #288,[4] and Methotrexate-resistant leukemia L1210.[4]

Manometric analysis.—Determination of glycolysis was carried out under anaerobic and/or aerobic conditions. The gas liberated in the former represents CO_2 evolved from acid formed by anaerobic glycolysis; the latter providing information on both aerobic glycolysis and respiration. Several experiments were carried out in Summerson differential manometers, which enabled direct determination of aerobic glycolysis, O_2 uptake, and CO_2 liberation of respiration. This has been described in a previous publication (*2*).

Results

The results of incubating human leukemic cells with various hexose analogues are summarized in table 1. The 1st- and 3rd-hour manometric readings are given as examples. The study on C. K., an adult with chronic lymphocytic leukemia, illustrates the effects of these 6 sugar analogues on leukocyte glycolysis. Anaerobic glycolysis was markedly inhibited by incubating with 2-DG and 2-D-Gal and these effects were noted within minutes after starting the incubation.

The degree of inhibition obtained was a function of the concentration of the agent used which ranged from 0.5 to 5.0 mg. per ml., and it also depended upon the amount of glucose present. In cells from C. K., a 63 percent inhibition took place with 2-DG at 1.0 mg. per ml., a 69 percent with 2.5 mg. per ml., and an 80 percent glycolytic inhibition with 5.0 mg. per ml. This compared to a 66 percent inhibition with 5.0 mg. of 2-D-Gal at 3 hours. Additive effects resulted when cells were incubated in the presence of both 2-DG and 2-D-Gal. Addition of 3-*O*-methyl-D-fructose and 6-*O*-methyl-D-glucose had no significant inhibitory effects: 2.5 mg. of 6-deoxy-6-fluoro-D-glucose had an equivocal effect. The glucose concentrations fell and eventually became rate limiting in all vessels, first in the actively glycolyzing vessels and last in those inhibited by 2-DG and 2-D-Gal, the two latter glycolyzing initially at low rates and therefore persisting much longer before glucose was depleted.

The inhibitory effects of graded quantities of 2-DG and 2-D-Gal on

[4] Kindly provided by Drs. Lloyd Law and Michael Potter.

TABLE 1.—*Effects of glucose analogues on glycolysis by human leukemic cells* in vitro (*mm. pressure change*)

Patient, diagnosis,* and date	Gas phase	Hour	Control	Compound added (*mg./ml.*)†																	
				2-DG					2D-Gal				2-DG +2D-Gal		6F-Gluc			6M-Gluc	3M-Fruct	3M-Gluc	3D-Gluc
				0.5	1.0	2.5	5	10	0.5	2.5	5	10	2.5 each	5 each	2.5	5	10	5	5	5	5
R.R. A.M.L. 3/15/57	5% CO_2/air	1 3 5	37 31 27				6 6 5				0 0 0			−1 3 1		27 26 25		39 27 24	28 27 23	37 29 26	32 26 28
E.R. C.M.L. 2/28/57	5% CO_2/N_2 5% CO_2/air	1 3 1 3	192 75 53 58			77 57 18 14	60 42 5 5	27 14 5 1		142 114 43 21	129 91 4 −2	130 82 7 −2	69 50 9 −1	45 32 −4 −4	108 93 52 32		83 66 22 14	166 78			
C.K. C.L.L. 5/17/57	5% CO_2/N_2	1 3	79 68		40 25	33 21	23 13				49 23		23 17		57 50			68 57	90 76		
M.P. A.M.L. 3/15/57	5% CO_2/air	1 3	45 45	−2 0							−3 −2					16 12			37 38		
D.U. A.L.L. 6/4/57	5% CO_2/N_2 5% CO_2/air	1 3 1 3	35 37 3 4	25 16 5 −2			15 1 8 −7		43 27 3 −4		22 10 −4 −6				37 46 12 0				47 35 14 4		
D.U. A.L.L. 5/29/57	5% CO_2/N_2 5% CO_2/air	1 3 1 3	84 66 −2 4	57 31 −16 −8		21 14 −10 −10	15 6 −6 −10		66 44 −2 3		42 27 −8 −8										
S.O. A.L.L. 6/7/57	5% CO_2/N_2	1 3	23 24	18 17			8 3		30 9		4 1								25 26		

C.B. Leukocytosis 5/8/57	5% CO_2/N_2	1 3	35 31				18 14	18 15														
W.R. A.Mono.L. 6/2/57	5% CO_2/N_2	1 3	21 18	13 13			1 3															
	5% CO_2/air	1 3	9 13	12 10			10 2															
J.S. A.L.L. 6/19/57	5% CO_2/N_2	1 3	66 70	49 39			9 9															
	L.A. μg./ml.		536	420			242															
W.R. A.Mono.L. 6/25/57	5% CO_2/N_2	1 3	30 33			7 6																
	L.A. μg./ml.		1032			69																
	5% CO_2/air	1 3	23 23			7 5																
	L.A. μg./ml.		529			75																

*A.M.L. = acute myelocytic leukemia; C.M.L. = chronic myelocytic leukemia; C.L.L. = chronic lymphocytic leukemia; A.L.L. = acute lymphocytic leukemia; A.Mono.L. = acute monocytic leukemia; L.A. = lactic acid.

†2-DG = 2-deoxy-D-glucose; 2D-Gal = 2-deoxy-D-galactose; 6F-Gluc = 6-deoxy-6-fluoro-D-glucose; 6M-Gluc = 6-*O*-methyl-D-glucose; 3M-Fruct = 3-*O*-methyl-D-fructose; 3M-Gluc = 3-*O*-methyl-D-glucose; 3D-Gluc = 3-deoxy-D-glucose.

aerobic and anaerobic glycolysis were noted in an experiment performed on E. R., a patient with chronic myelogenous leukemia. In this study 6-deoxy-6-fluoro-D-glucose also caused inhibition where 6-*O*-methyl-D-glucose did not. In cells taken from R. R., a 10-year-old boy with acute myelogenous leukemia, it was found that 2-DG and 2-D-Gal profoundly inhibited aerobic glycolysis, although the same quantities of 3-*O*-methyl-D-glucose, 3-*O*-methyl-D-fructose, 6-*O*-methyl-D-glucose, and 6-deoxy-6-fluoro-D-glucose had virtually no effect on the over-all aerobic reading. In the studies on cells from D. U., a 6-year-old boy with acute lymphocytic leukemia, significant aerobic and anaerobic glycolytic inhibition was achieved by 0.5 mg. 2-DG and 2-D-Gal per ml. of cell suspension. With W. R., a 60-year-old Negro with acute monocytic leukemia, the inhibition of glycolysis caused by 2-DG was reflected as a decreased amount of lactic acid production, from 529 μg. per ml. in the controls to 75 μg. per ml. in the experimental vessels. Studies on S. O. and J. S., both children with acute lymphocytic leukemia, demonstrated similar glycolytic inhibitions as described. In C. B., a man with benign leukocytosis, 5.0 mg. of 2-DG caused a 55 percent glycolytic inhibition at 3 hours; increasing the concentration had no enhancing effect.

Three mouse tumor types in ascites form were studied in this system. The results are summarized in table 2. With Krebs-2 ascites, aerobic and anaerobic glycolysis were markedly inhibited in the 1st hour by 2-DG. Glycolysis of Lymphocytic neoplasm #288 was inhibited by 2-DG, 2-D-Gal, and 6-deoxy-6-fluoro-D-glucose. In the Methotrexate-resistant strain of mouse leukemia L1210, 2-DG and 2-D-Gal gave potent glycolytic inhibition.

Table 3 summarizes an experiment on administration of 2-deoxy-D-glucose *in vivo* to a patient with chronic myelogenous leukemia. Graded amounts of glucose were added to the control sample and to the sample taken 5 minutes after the end of infusion in order to approximate the glucose concentration in the samples drawn at 2 and 4 hours after infusion.

TABLE 2.—*Effect of glucose analogues on glycolysis by mouse tumor cells* in vitro (*mm. pressure change*)

Tumor and date	Gas phase	Hour	Control	Compound added (*mg./ml.*)					
				2-DG		2D-Gal		3M-Gluc	6F-Gluc
				2.5	5	2.5	5	5	5
Krebs-2 ascites 3/22/57	5% CO_2/N_2	1	144		78				
	5% CO_2/air	1	82		27				
		2	81		40				
Lymphocytic neoplasm #288 3/29/57	5% CO_2/N_2	1	123		22	91		86	8
		3	92		10	60		84	32
Methotrexate-resistant leukemia (L1210) 3/21/57	5% CO_2/air	1	33	17	5	6	6		
		3	23	8	3	4	2		

TABLE 3.—*Effect of 2-deoxy-D-glucose infusion on leukemic cell glycolysis**

	Control	Five minutes after infusion	Two hours after infusion	Four hours after infusion
Initial glucose mg. percent	83	99	137	192
Glucose added *in vitro* mg. percent	80	40	0	0
Total glucose present mg. percent	163	139	137	192
Millimeter gas produced per hour (for 185,000 cells/mm.^{3})	131	86	82	95
Millimeter gas produced per hour per 10 million cells	6.6	4.3	4.1	4.8

*Sixty mg. per kg. infused to patient with chronic myelogenous leukemia over 1-hour period. Blood drawn sequentially for incubation.

Aerobic readings, directly reflecting aerobic glycolysis, were reduced from 6.6 mm. gas per hour per 10 million cells to 4.3 mm. 5 minutes after the infusion. Glycolytic inhibition was present even 4 hours after infusion at a time when 2-deoxy-D-glucose had fallen from initial values of 8 mg. percent to 2 mg. percent. All the manometric results are given as the average of triplicate determinations.

In another experiment on a patient with acute myelogenous leukemia, a 40 percent inhibition of glycolysis was observed after administration of 2-deoxy-D-glucose at 50 mg. per kg. Two other such experiments gave equivocal results, at the relatively low doses employed.

Discussion

Hexose analogues substituted on the C-2 position have been shown to have a particular ability to serve as glycolytic inhibitors (*9*, *22*). These studies have extended observations with 2-DG to human leukocytes in the various leukemias. They have also established for the first time, 2-deoxy-D-galactose as a potent glycolytic inhibitor of human leukemic leukocytes and of mouse tumor cells in every instance tested. The degree of glycolytic inhibition was mainly a function of glucose/inhibitor ratio rather than a variable sensitivity of a specific leukemic cell type to an inhibitor. There is a considerable body of evidence which suggests that the site of this competitive inhibition is on the mitochondrial hexokinase in the case of cancer cells. This has been shown directly (*23*) by overcoming the glycolytic block of 2-DG on mouse melanoma mitochondria by the use of glucose-6-phosphate as substrate. However there is little doubt that a strong inhibition of the hexokinase reaction will also cause other metabolic blocks, *e.g.*, insulin lack.

All the patients with leukemia (*22*) had active disease at the time of study, and all had markedly elevated white-blood-cell counts. Some were untreated, some were undergoing or had completed courses of antimetabolite therapy, and some were terminal cases in acute blastic crises. It is interesting that little or no distinction could be made among these types of patients in regard to inhibition by effective sugar analogues. None of the cells were "resistant" to glycolytic inhibition despite previous ex-

posure to antimetabolites other than these analogues. This was also evident in the animal tumor cell studied.

The glycolytic inhibition seen *in vitro* was not solely the function of the *in vitro* system, as indicated by the experiments of *in vivo* administration of 2-DG. Under such conditions, cell metabolism was altered while the cells were still within the body.

Serial sampling and metabolic study of leukemic leukocytes after the administration of a drug seems to be a feasible way of investigating changes induced in cancer cells. Indeed, use of these cells may make it possible to study their metabolic sensitivity to a variety of chemotherapeutic agents, to obtain information on *in vivo* alteration of cell metabolism following drug administration, and to investigate fat, protein, and carbohydrate metabolism at times of remission and active disease.

References

(*1*) LASZLO, J., STENGLE, J., WIGHT, K., and BURK, D.: Effects of chemotherapeutic agents on the metabolism of leukemic cells. (Abstract.) Proc. Am. Assoc. Cancer Res. 2: 224, 1957.

(*2*) ———: Effects of chemotherapeutic agents on the metabolism of human acute leukemia cells *in vitro*. Proc. Soc. Exper. Biol. & Med. 97: 127–131, 1958.

(*3*) BURK, D., LASZLO, J., STENGLE, J., and WIGHT, K.: Metabolism of human leukemic lymphoblasts and its normalization by methotrexate and 6-mercaptopurine. (Abstract.) Fed. Proc. 16: 160–161, 1957.

(*4*) BURK, D.: Über die begründung einer chemotherapie des Krebses auf der grundlage einer preimären hemmung der glucosephosphorylierung (hexokinasereaktion) durch beeinflussung von substrat, coenzym und enzym. Klin. Wchnschr. 35: 1102–1105, 1957.

(*5*) BURK, D., HOCHSTEIN, P., HUNTER, J., LANDAU, B., LASZLO, J., WIGHT, K., and WOODS, M.: A rationale of cancer chemotherapy based on primary inhibition of glucose phosphorylation (hexokinase reaction) at substrate, coenzyme, and enzyme levels. Acta Unio internat. contra cancrum 2466–2467, 1958.

(*6*) LASZLO, J., LANDAU, B., WIGHT, K., and BURK, D.: Anticancer-effects of hexose sugar analogs. (Abstract.) Proc. Am. Assoc. Cancer Res. 2: 318, 1958.

(*7*) BURK, D., WIGHT, K., and WOODS, M.: Insulin: anti-insulin regulation of metabolism in selective mechanisms of cancer chemotherapy, among a spectrum of malignancies. (Abstract.) Proc. Am. Assoc. Cancer Res. 2: 285, 1958.

(*8*) BURK, D., LASZLO, J. WIGHT, K., and WOODS, M.: The metabolic basis of cancer chemotherapy. (Abstracts.) Seventh Internat. Cancer Congress, London, 235–236, 1958.

(*9*) WOODWARD, G. E., and HUDSON, M. T.: The effect of 2-desoxy-D-glucose on glycolysis and respiration of tumor and normal tissues. Cancer Res. 14: 599–605, 1954.

(*10*) WOODWARD, G. E.: 2-Desoxy-D-glucose as an inhibitor in the aerobic glucose metabolism of yeast. J. Franklin Inst. 254: 553–555, 1952.

(*11*) ELY, J. O., TULL, F. A., and HARD, J. A.: The influence of 2-desoxy-D-glucose on the growth of embryonic chicken heart fibroblasts in tissue culture. J. Franklin Inst. 253: 361–365, 1952.

(*12*) ELY, J. O.: 2-Deoxy-D-glucose as an inhibitor of cancerous growth in animals. J. Franklin Inst. 258:157–160, 1954.

(*13*) BALL, H. A., WICK, A., and SANDERS, C.: Influence of glucose antimetabolites on the Walker tumor. Cancer Res. 17: 235–239, 1957.

(*14*) Sokoloff, B., Eddy, W. H., Saelhof, C. C., and Beach, J.: Glucose antagonists in experimental cancer. A. M. A. Arch. Path. 59: 729–732, 1955.

(*15*) Laszlo, J.: Unpublished data.

(*16*) Wick, A. N., Drury, D. R., Nakada, H. I., and Wolfe, J. B.: Localization of the primary metabolic block produced by 2-deoxyglucose. J. Biol. Chem. 224: 963–969, 1957.

(*17*) Overend, W. C., Shafezadek, F., and Stacey, M.: An investigation of the glycal method for the preparation of derivatives of 2-deoxy-D-galactose. J. Chem. Soc. 992–993, 1951.

(*18*) Glen, W. L., Myers, G. S., and Grant, C. A.: Monoalkyl hexoses: improved procedures for the preparation of 1- and 3-methyl ethers of fructose and of 3-alkyl ethers of glucose. J. Chem. Soc. 2568–2572, 1951.

(*19*) Bell, D. J.: Crystalline 6-methylglucose, a new synthesis. J. Chem Soc. 859–861, 1936.

(*20*) Cramer, F. B., and Neville, C. A.: A colorimetric method for estimating 2-desoxyglucose. J. Franklin Inst. 256: 379–381, 1953.

(*21*) Barker, S. B., and Summerson, W. H.: The colorimetric determination of lactic acid in biological material. J. Biol. Chem. 138: 535–554, 1941.

(*22*) Landau, B., Laszlo, J., Stengle, J., and Burk, D.: Certain metabolic and pharmacologic effects in cancer patients given infusions of 2-deoxy-D-glucose. J. Nat. Cancer Inst. 21: 485–494, 1958.

(*23*) Hochstein, P.: Unpublished data.

Certain Metabolic and Pharmacologic Effects in Cancer Patients Given Infusions of 2-Deoxy-D-Glucose [1]

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Summary

2-Deoxy-D-glucose (2-DG) was administered to 1 patient with islet-cell carcinoma, 5 patients with leukemia, 1 patient with bronchogenic carcinoma, and 1 with renal-cell carcinoma. After a single intravenous infusion of 2-DG, the following effects were noted: 1) Blood-glucose levels rose. 2) Symptoms were diaphoresis, generalized warmth, flushing, headache, drowsiness, and tachycardia. No symptoms were severe and all of them were transient. 3) In the leukemic patients, white-blood-cell counts either fell directly, or rose and then fell during the 24-hour period following administration. 4) Glycolytic inhibition of leukemic cells occurred in some of the patients. 5) Approximately 30 percent of the administered dose was excreted in the urine; the fate of the remainder of the compound was unknown. The significance of these observations was discussed. **J. Nat. Cancer Inst. 21: 485–494, 1958.**

The requirement of cancer cells for glucose, whatever the underlying basis for this metabolic demand, offers a possible attack site for cancer chemotherapy (*1–3*). Many glucose analogues have therefore been investigated as potential glucose antimetabolites (*2*); of these, 2-deoxy-D-glucose (2-DG) has, perhaps, proved the most promising. 2-DG has been reported to inhibit growth of experimental mouse and rat tumors (*4–6*). It has been shown regularly to inhibit glycolysis of mouse leukemic cells, human leukocytes (*7*), and Krebs-2 ascites carcinoma cells (*7, 8*) *in vitro*. Blood-glucose levels rise after 2-DG administration to rats, rabbits, cats, and dogs (*9–11*), observations consistent with its action as a glucose antagonist. Because of its anti-animal tumor effects *in vivo*

[1] Received for publication February 13, 1958.

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[5] The authors are grateful to Dr. Roy Hertz and Dr. Emil Frei for their helpful suggestions.

[6] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

and *in vitro* and its ability to raise blood-glucose levels in animals, 2-DG administration to cancer patients has been initiated. Thus far, 8 patients have received intravenous infusions of 2-DG; of these, 5 will be described in detail in this report. One patient studied had islet-cell carcinoma with hyperinsulinism, 1 had bronchogenic carcinoma, 2 had acute leukemia, and 1 had chronic lymphocytic leukemia.

Materials and Methods

2-DG obtained from the Aldrich Chemical Company, Milwaukee, Wisconsin, had been recrystallized from amyl alcohol after treatment with animal charcoal, and then had been dried *in vacuo*. It was a white, odorless powder, with a minimum purity of 99 percent by analysis, demonstrating only one component on chromatography, and melting at 149° C. (reported 148°), (*12*). 2-DG was administered as a 20 percent aqueous solution after Seitz filtration and pyrogen testing.

The patients in these studies were fasted but were given water for a 12-hour period prior to 2-DG administration and throughout the experimental period. The sole exception to this procedure was C. K., the patient with hyperinsulinism, who had to be maintained on 30 gm. glucose hourly as a 10 percent solution given intravenously by means of a constant infusion pump. 2-DG was administered intravenously during a 25-minute to 1-hour period at doses ranging from 50 mg. per kg. to 200 mg. per kg. The patients were under constant observation with vital signs taken at frequent intervals. EKG's were recorded for most patients and an EEG in 2 instances. Frequent white-blood-cell counts were taken of patients who had leukemia.

Blood samples were taken at intervals to determine the blood-glucose (*13*) and blood 2-DG (*14*) levels. Urine for 2-DG determinations was collected from patients (C. K. and M. P.) with indwelling urinary bladder catheters, at hourly intervals, and from the other patients at 2-hour intervals. Blood samples for metabolic studies of leukocytes were taken from the patients with leukemia before, immediately following, and several hours after 2-DG administration (*7*).

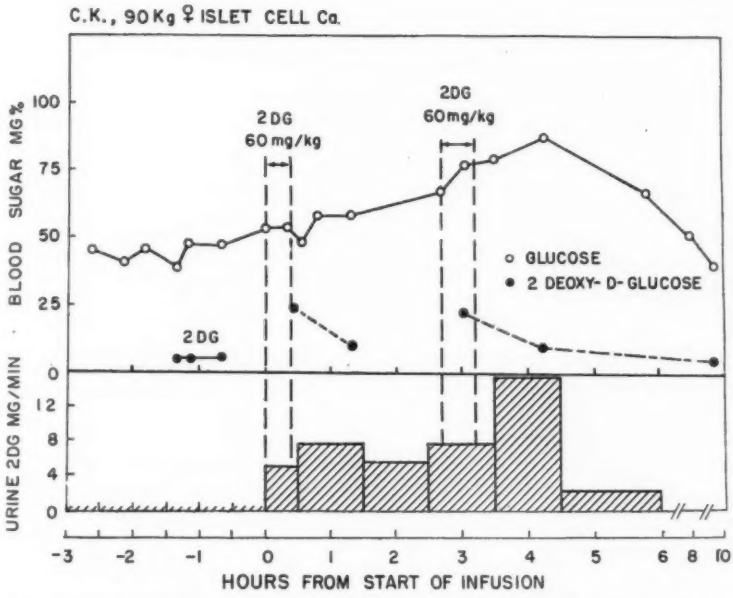
Glucose utilization tests were made after intravenous glucose (25 gm.) was given, as described by other workers (*15*). In these studies the patient was fasted for 12 hours before receiving a 2-DG infusion in saline (60 mg./kg.), or an oral 2-DG (60 mg./kg.). Both oral and intravenous 2-DG had been given in previous experiments to determine the blood-glucose curve in order to predict at what point of maximum hyperglycemia the glucose would be administered. Blood samples were taken every 8 minutes after glucose load and the percent of disappearance per minute (K) calculated (*15*).

Clinical Studies and Results

Patient C. K.—A 28-year-old Negro woman admitted to the National Cancer Institute had a history of recurrent hypoglycemic episodes of 2 months' duration. A large islet-cell tumor of the pancreas with exten-

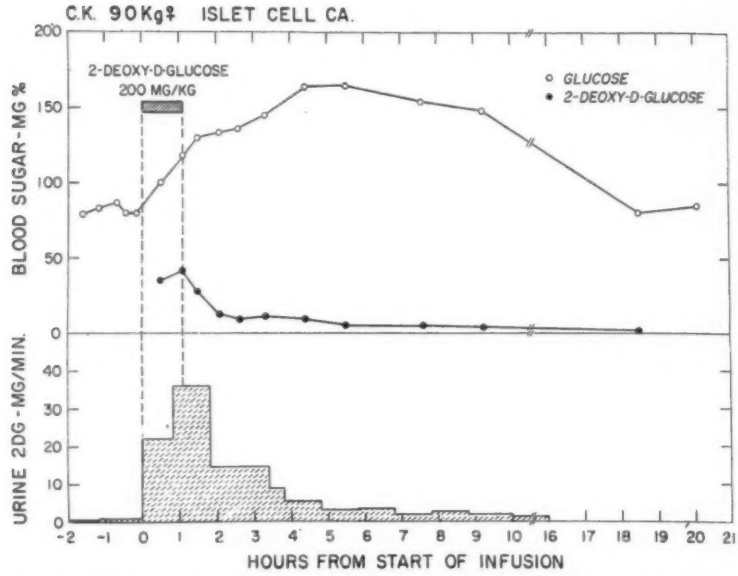
sive liver metastases was demonstrated at laparotomy. Removal of the primary tumor did not result in clinical improvement, and the patient continued to require large quantities of intravenous glucose solutions infused constantly to maintain a near normal, blood-glucose level. There was no clinical or laboratory evidence of heart, liver, or kidney damage.

The patient first received 60 mg. per kg. body weight of 2-DG during a half-hour period after several control blood-sugar determinations had been made (text-fig. 1). No significant rise in blood-glucose level was observed, and except for a marked diaphoresis immediately after completion of the 2-DG administration, no other response was evident. Another 60 mg. per kg. dose was infused and then there was a rise in blood-glucose



TEXT-FIGURE 1.—Response of patient C. K. on infusions of 2-DG (60 mg./kg.).

level, but this rise persisted only a short time. 2-DG levels were high immediately after 2-DG administration, but rapidly decreased. During the 6½-hour period after the onset of 2-DG administration, 24 percent of the dose was excreted in the urine. Six days later, 200 mg. per kg. of 2-DG was infused over a 1-hour period. Blood-glucose levels rose rapidly to almost twice the control values and remained elevated for at least 9 hours (text-fig. 2). Thirty percent of the dose was excreted in the urine during a 16-hour period, most of the excretion occurring within the first few hours after the beginning of 2-DG administration. The symptoms experienced by the 5 patients are listed in table 1. Chief manifestations of 2-DG administration to patient C. K. were feelings of warmth, drowsiness, and a general diaphoresis. These appeared toward the end



TEXT-FIGURE 2.—Response of patient C. K. on infusion of 2-DG (200 mg./kg.).

TABLE 1.—*Symptoms of patients after 2-DG administration*

Patient	Diagnosis	Dose (*mg./kg.*)	Flush	Warmth	Diaphoresis	Headache	Drowsiness	Hypothermia
C. K.	Islet-cell carcinoma	120 200	0 0	3+ 3+	4+ 3+	0 0	2+ 2+	1+ 1+
M. P.	Acute myelogenous leukemia	50	4+	4+	4+	0	2+	3+
M. K.	Chronic lymphocytic leukemia	50	0	4+	4+	0	2+	0
D. U.	Acute lymphocytic leukemia	50 50 50	0 0 0	2+ 3+ legs only	0 1+ 0	2+ 0 2+	1+ 4+ 0	0 ± 0
S. B.	Bronchogenic carcinoma	90 60 60 (oral)	2+ 2+ 0	4+ 3+ 1+	4+ 4+ 2+	0 0 0	3+ 2+ 0	3+ 2+ 1+

of the infusion and persisted for 30 minutes. The subjective effects were transient and there was no detectable change in the course of her fatal illness.

Patient M. P.—This 59-year-old white woman was admitted to the National Cancer Institute on March 15, 1957, with a diagnosis of acute myelogenous leukemia. A month earlier she had complained of an upper respiratory infection associated with progressive weakness, fatigue, and

anorexia. Her white-blood-cell count was 300,000 cells per mm.^{3}, 57 percent of which were myeloblasts. Bone-marrow cells were 99 percent in the granulocytic series of which 52 percent were myeloblasts. Lymphadenopathy and hepatosplenomegaly were noted. The patient was started on combined therapy with Methotrexate (Amethopterin) and 6-mercaptopurine, which was followed by partial clearing of blast cells from the peripheral blood and marked subjective improvement. However, on March 25, she developed paralysis of the left leg, a left facial palsy, and retinal hemorrhages. On March 31, Meticorten (Prednisone) therapy was begun, and on April 2, Methotrexate and 6-mercaptopurine were discontinued because of a rise of BUN (blood urea nitrogen) to 33. On April 5, 2-DG was infused (50 mg./kg.), while Meticorten was discontinued just before and during the study. A 30 percent rise in blood glucose, following the infusion, was observed with about 30 percent of the administered dose of 2-DG excreted during the first 3 hours. Blood levels of 2-DG after the initial determination were 12 mg. percent or less, and decreased slowly.

At the end of the infusion the patient had a feeling of warmth, marked diaphoresis, and her rectal temperature fell from 38° to 35° C. Her face was flushed for almost 2 hours and was reminiscent of that seen in patients with carcinoid tumors (*16*). However, urinary 5-hydroxytryptamine levels were within normal limits.[7] Vital signs were stable except for a lowering of diastolic pressure and a short period of tachycardia. Continuous EKG's showed a brief period during which the occurrence of premature ventricular beats was more frequent than on control tracings.

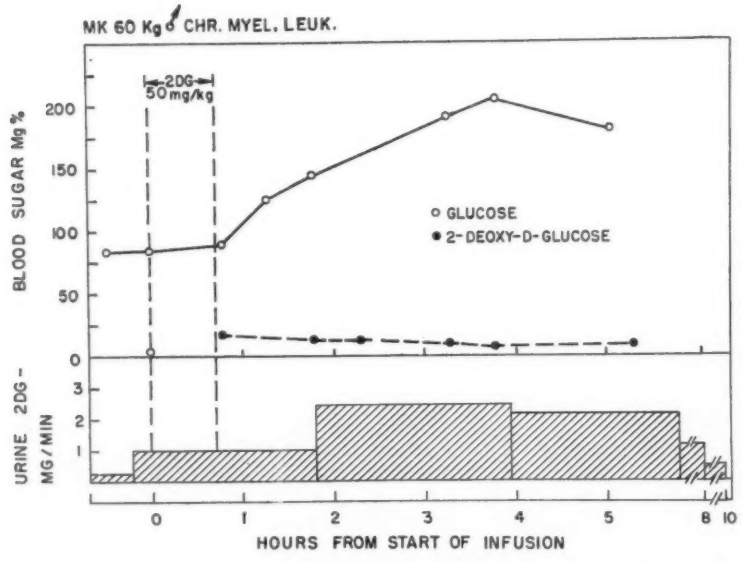
Leukemic leukocytes, removed 2 hours after completion of 2-DG infusion, glycolyzed at 50 percent of the rate determined in the control sample. The drop in white count from 74,000 to 38,000 per mm.^{3} was complicated by initiation of Methotrexate and 6-mercaptopurine therapy 16 hours prior to determination of the final count. However, in succeeding days, despite the continued use of these medications, the white count rose as steadily as it had before 2-DG administration. The patient died on April 12 of recurrent brain hemorrhage.

Patient M. K.—This 50-year-old man had generalized lymphadenopathy and hepatosplenomegaly. His white-blood-cell count was 304,000 with 95 percent of the cells in the lymphocytic series. Bone-marrow aspiration was diagnosed as chronic lymphocytic leukemia. On May 17, 1957, M. K. was admitted to the National Cancer Institute for further examination and possible therapy. Laboratory data included a hemoglobin of 7 gm. percent, a white-blood-cell count of 204,000 cells per mm.^{3} with 97 percent lymphocytes which had 5 percent abnormal forms, and a platelet count of 38,000 per mm.^{3} BUN, liver chemistries, and urinalysis were within normal limits. 2-DG infusion (50 mg./kg.) caused a rise in blood glucose from controls of 80 mg. percent to over 200 mg. percent (text-fig. 3). At peak blood glucose, a trace of glycosuria was present. 2-DG excretion was 25 percent of the administered dose over a 10-hour

[7] 5-Hydroxytryptamine determinations were by courtesy of Dr. Paul Condit of the National Cancer Institute.

period. The patient experienced side effects very similar to those of M. P. (table 1). Glycolysis of cells taken from M. K. was slightly inhibited anaerobically at 3 hours. White blood count initially rose, and then fell back toward control levels. No EKG abnormalities were noted. There was no demonstrable change in the patient's clinical course and he later responded to Meticorten therapy and was discharged.

Patient D. U.—In January, 1956, this 7-year-old white boy developed pallor, anorexia, and vomiting, with dyspnea on exertion. Physical and laboratory examinations revealed acute lymphocytic leukemia. He was treated with cortisone and Methotrexate which was followed by a complete remission but he had a relapse in May, 1956. Another course of Methotrexate, followed by treatments with 6-azauracil and 6-mercapto-

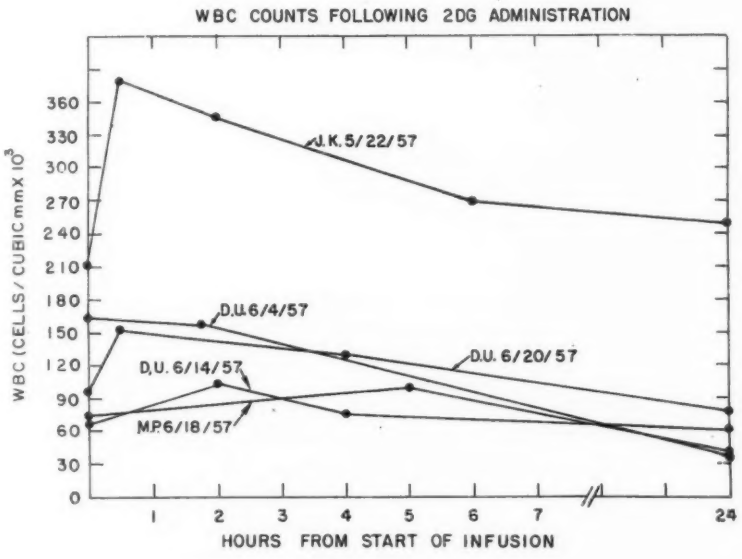


TEXT-FIGURE 3.—Response of patient M. K. on infusion of 2-DG (50 mg./kg.).

purine, was without benefit. In January, 1957, a severe *Pseudomonas aeruginosa* infection of the left knee developed. The infection was treated with antibiotics and Meticorten. In February, 1957, he received 6-azathymine without apparent benefit, but in March he spontaneously improved. He was readmitted to the National Cancer Institute on May 27, 1957, in a relapse. Laboratory data included a hemoglobin of 10.8 gm., a platelet count of 22,000, and a white-blood-cell count of 56,000, with 93 percent of the cells as abnormal lymphocytic forms. A BUN was 13 mg. percent; alkaline-phosphatase, cephalin flocculation and BSP tests were normal. Leukemic infiltration of bone was demonstrated by X ray. Treatment with 6-azathymine was initiated and continued until June 5. On June 4, 1957, 2-DG was administered and the treatment was repeated on June 13 and June 20. Blood-glucose levels rose 40 percent higher than

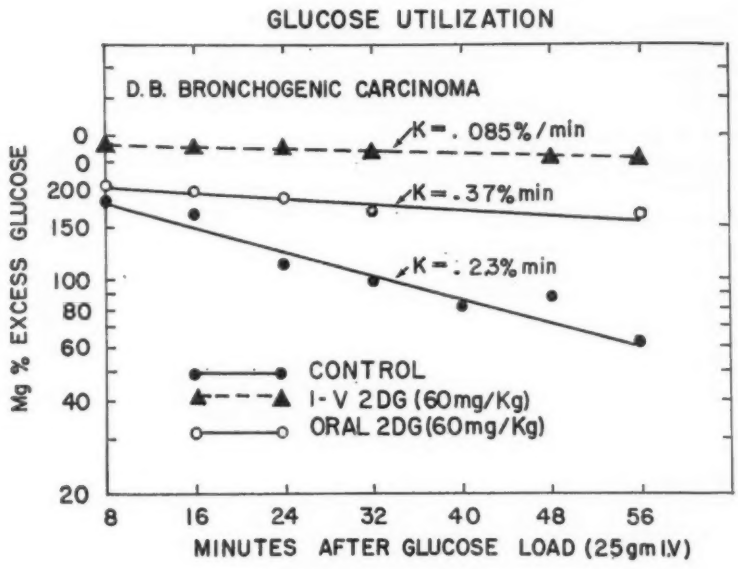
control levels. Blood 2-DG levels, urinary excretion, and side effects were similar to those described for M. P. Continuous EEG's and EKG's were unchanged during infusion. Changes in white-blood-cell counts are shown in text-figure 4. The pattern was either a direct fall or a rise followed by a fall in white count.

Patient S. B.—This 54-year-old male, a chronic smoker, had a diagnosis of bronchogenic carcinoma made in July, 1957, following a persistent cough, weakness, and dyspnea on exertion. He was treated with nitrogen-mustard therapy in September, 1957, and continued on this medication after being hospitalized at the National Cancer Institute on September 30, 1957. Glucose utilization studies were made on October 30, October 31, and November 2, and he was given 2-DG prior to the second and third studies.



TEXT-FIGURE 4.

In 3 studies on S. B., the oral 2-DG (60 mg./kg.) raised blood glucose from 109 mg. percent to a maximum of 180 mg. percent; intravenous 2-DG at 60 mg. per kg. raised blood glucose from 95 mg. percent to a maximum of 198 mg. percent; intravenous 2-DG at 90 mg. per kg. raised blood glucose from 85 mg. percent to 250 mg. percent. The administration of oral or intravenous 2-DG markedly inhibited glucose utilization, as shown in text-figure 5. Oral 2-DG at 60 mg. per kg. reduced the glucose utilization from control values of 2.3 percent per minute to 0.37 percent per minute; intravenous 2-DG reduced this to 0.085 percent per minute. When a correction was made for the glucose excreted in the urine during the test period, a value approaching zero was obtained.



TEXT-FIGURE 5.

Side effects noted in these studies were similar to those previously described for other patients. An additional study was made with a dose of 2-DG at 90 mg. per kg. under continuous EEG and EKG observations. No changes were noted even at the time of the patient's drowsiness. All side effects except hypothermia lasted for less than 90 minutes and there was no change in the course of the disease. The patient had failed to respond to nitrogen mustard and was under treatment with Methotrexate.

2-DG was also administered to P. S., a 59-year-old man with metastatic renal-cell carcinoma, and to D. B., a 22-year-old man, and to C. F., a 62-year-old man, both with chronic myelogenous leukemia. Studies of blood glucose, blood 2-DG, glucose tolerance tests, and side effects in these patients were similar to those noted in other patients and will not be described in detail. Glycolysis of leukemic leukocytes taken from C. F. were inhibited 35 percent 5 minutes after the end of the infusion and remained at approximately that level for the 3-hour period studies.

Discussion

As in animals, 2-DG in humans causes a rise in blood glucose. This blood-glucose rise does not appear related to epinephrine release, as evidenced by the fact that no rise in blood pressure was observed and by the prolonged nature of the glucose elevation. C. K. required larger doses of 2-DG for a significant blood-glucose rise than the other patients, which may be related to the large quantities of glucose C. K. was receiving and may thus be a reflection of the competitive nature of the 2-DG block.

The side effects noted at the doses used were transient and not severe.

Flushing, sweating, and drowsiness were suggestive of a hypothalamic response. No neurologic behavior such as that observed in animals (*9*, *10*) given high doses of 2-DG was noted in these patients. Sweating and drowsiness could also be the result of inadequate glucose utilization by brain cells causing symptoms like those seen in hypoglycemic reactions. However, the patients did not complain of hunger, were not confused, and did not show any convulsive phenomena. EEG's in 2 patients were unchanged by 2-DG administration.

About 30 percent of the 2-DG administered could be accounted for in the urine when measured as quinaldine positive material. Little of the 2-DG was excreted in the urine at the time the studies were completed and the fate of the remainder of the compound is unknown. 2-DG is phosphorylated to 2-deoxy-D-glucose-6-phosphate, but this is not a substrate for phosphohexoisomerase or glucose-6-phosphate dehydrogenase (*17*, *18*), so that once formed, it should not be metabolized further. Support for this is found in the failure to isolate radioactive carbon dioxide from rabbits that were eviscerated and nephrectomized and given radioactive 2-DG (*18*). Perhaps the 2-DG phosphate is only slowly dephosphorylated and excreted.

No adequate explanation is as yet available for the changes in white blood-cell count consisting of a fall, or a rise followed by a fall, during the 24-hour period after 2-DG administration. This has been repeated with other leukemic patients, not described here, in whom saline control experiments produced no appreciable changes in white blood count. It should be noted that orally administered 2-DG was well absorbed and produced metabolic changes and side effects equivalent to a somewhat lower dose of 2-DG given intravenously. Both routes of administration markedly interfered with glucose utilization as measured by the handling of glucose loads.

Because of the potent metabolic effects of 2-DG after a single intravenous infusion, studies of repeated administration of 2-DG to cancer patients have been instituted.

References

(*1*) Burk, D.: Über die begründung einer chemotherapie des Krebses auf der grundlage einer primären hemmung der glucosephosphorylierung (hexokinase-reaktion) durch beeinflussung von substrat, coenzym und enzym. Klin. Wchnschr. 35: 1102–1105, 1957.

(*2*) Woods, M., Hunter, J., and Burk, D.: Regulation of glucose utilization in tumors by a stress-modified insulin:anti-insulin system. J. Nat. Cancer Inst. 16: 351–404, 1955.

(*3*) McDonald, E., and Cramer, F. B.: Cancer research. J. Franklin Inst. 253: 351–353, 1952.

(*4*) Ball, H. A., Wick, A. N., and Sanders, C.: Influence of glucose antimetabolites on the Walker tumor. Cancer Res. 17: 235–239, 1957.

(*5*) Laszlo, J., Landau, B., Wight, K., and Burk, D.: Anticancer effects of hexose sugar analogs. (Abstract.) Proc. Am. Assoc. Cancer Res. 2: 318, 1958.

(*6*) Sokoloff, B., Saelhof, L., Kato, S., Chamelin, I., and Beach, J.: Effect of a glucose analog on nucleic acids in tumor tissue. Growth 20: 265–273, 1956.

(7) LAZLO, J., LANDAU, B., WIGHT, K., and BURK, D.: The effect of glucose analogues on the metabolism of human leukemic cells *in vitro*. J. Nat. Cancer Inst. 21: 475–483, 1958.

(8) YUSHOK, W. D., and BATT, W. G.: D-glucose analogs with substituted groups on the number two carbon. Effects on metabolism of ascites tumor cells. *In* Abstracts, Am. Chem. Soc. 131st Meeting, 1957, p. 120.

(9) LANDAU, B. R., and LUBS, H. A.: Animal responses to 2-deoxy-D-glucose administration. In press.

(10) MORRELL, R. M., and LANDAU, B. R.: Effect of 2-deoxy-D-glucose on cerebral metabolism in cats. Unpublished data.

(11) LANDAU, B. R., SHIP, A., and LEVINE, H. J.: Action of insulin on distribution of glucose analogs in eviscerated-nephrectomized dogs. Am. J. Physiol. 193: 461–465, 1958.

(12) BERGMANN, M., SCHOTTE, H., and LECHINSKY, W.: Über die ungesattigten reduktions produkte der zuckerarten und ihre umwandlugen, III.: Über 2-desoxy-glucose. Ber. Deut. Chem. Geo. 55: 158–172, 1922.

(13) NELSON, N.: A photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem. 153: 375–380, 1944.

(14) CRAMER, F. B., and NEVILLE, G. A.: A colorimetric method for estimating 2-desoxyglucose. J. Franklin. Inst. 256: 379–381, 1953.

(15) MARKS, P. A., and BISHOP, J. S.: The glucose metabolism of patients with malignant disease and of normal subjects as studied by means of an intravenous glucose tolerance test. J. Clin. Invest. 36: 254–264, 1957.

(16) SJOERDSMA, A., WEISSBACH, H., TERRY, L. L., and UDENFRIEND, S.: Further observations on patients with malignant carcinoid. Am. J. Med. 23: 5–15, 1957.

(17) SOLS, A., and CRANE, R. K.: Substrate specificity of brain hexokinase. J. Biol. Chem. 210: 581–595, 1954.

(18) WICK, A. N., DRURY, D. R., NAKADA, H. I., and WOLFE, J. B.: Localization of the primary metabolic block produced by 2-deoxyglucose. J. Biol. Chem. 224: 963–969, 1957.

Quantitative Evaluation of Chemotherapeutic Agents Against Advanced Leukemia in Mice [1]

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Summary

An assay system employing advanced systemic leukemia (L1210) in mice has been utilized to compare the antileukemic effectiveness of a series of chemical agents. Comparison was made of the relative effectiveness of the compounds in increasing the survival time of the mice, employing the folic acid analogue amethopterin as a standard. Data on 38 compounds are presented. Twenty-three of these had been previously studied against early leukemia and in other tumor systems in a comprehensive study of screening (*4*). None of the compounds used was as effective as amethopterin in increasing the survival time of the leukemic mice. Reserpine, 6-mercaptopurine, thioguanine, 8-azaguanine, and 5-fluorouracil were 50 to 60 percent as effective as amethopterin. Aminopterin, 2 thiadiazole derivatives, azaserine, and 3 pyrimidines showed 30 to 50 percent of the activity of amethopterin. The remaining compounds tested showed lesser or no activity. The application of this type of assay procedure as a tool for screening and the quantitative evaluation of the action of potential antineoplastic agents are discussed.
J. Nat. Cancer Inst. 21: 495–511, 1958.

Following the introduction of systematic antitumor-screening programs in 1944 (*1*), there has been continued interest in the development of new methods for both screening and biological assay of candidate chemotherapeutic agents. The methodology employed in current antineoplastic screening procedures has been amply reviewed in previous publications (*2–4*). Formal recognition of the importance of problems pertaining to screening was forthcoming with the publication of the Gellhorn-Hirschberg report on "Investigation of Diverse Systems for Cancer Chemotherapy Screening" (*4*). The latter study was devoted to an examination of the methodology for the selection of antitumor compounds. The screening systems included experimental tumor systems based on a representative

[1] Received for publication February 14, 1958.
[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

spectrum of 15 tumors; microbiological systems; systems involving differentiation and development; and systems involving biochemical synthesis. In all, there was a total of 74 screening systems and 27 compounds were employed in each of them.

The following conclusions were drawn (*4*): *a*) None of the non-tumor systems was capable of replacing tumor systems as screening tools for carcinostatic agents. *b*) There was considerable variability of response to the chemotherapeutic agents among the various experimental neoplasms. No single tumor would have selected all of the useful agents, and the recommendation was made that the employment of a spectrum of tumors would be desirable in a screening system. *c*) It is difficult to compare antitumor activity in experimental and human neoplasms because of the lack of appropriate clinical data.

The complexity of the problem is further indicated by the observation in this laboratory that even in a single tumor system the antitumor effectiveness of a drug may be altered by factors pertaining to the host, the tumor, and the drug (*5–12*). The antileukemic action of folic acid analogues was, for example, influenced by such factors as the number of cells in the leukemic inoculum (*11*), the stage of the disease at which treatment was initiated (*7*), the age and weight of the host (*10*), and the use, and time of administration, of metabolites (*5*, *6*, *8*). Marked differences in antileukemic action were noted in accordance with the schedules of treatment (*7–9*, *12*). Thus, the activity rating of a drug, even for a specific tumor, may vary with the experimental conditions.

Despite these difficulties, with the advent of large-scale screening programs, the numbers of compounds that have shown activity in a primary screening program are increasing and may be expected to continue to increase. In addition, when a new active compound is found, a whole series of congeners may show varying degrees of activity.

These developments suggest that it may be desirable to have on hand procedures which (*a*) make it more difficult for a compound to show positive antitumor activity in a screen and which (*b*) permit a quantitative evaluation of the relative antitumor effectiveness of compounds so that appropriate selections may be made for further preclinical study.

One such assay system employing advanced systemic leukemia in mice was developed in this laboratory (*12*) after relatively successful therapy with amethopterin was observed early in the course of the disease (*13*). The assay employing the advanced leukemia has been applied here to obtain a comparison of the relative effectiveness of a series of antileukemic agents. Data on 23 of the compounds used in the Gellhorn-Hirschberg report (*4*) and 15 additional compounds are reported in the current study.

Methods

The general procedures are similar to those that have been utilized in previous studies (*12*, *14*). The tumor employed was Lymphoid leukemia L1210, an acute stem-cell leukemia originally induced by application of

methylcholanthrene (*15*), which has been carried in this laboratory in DBA mice (subline 212) for 9 years. The experiments were conducted with (BALB/cAn × DBA/2J)F_1 hybrid male mice of the same age (2 to 3 months) and weight (22 to 28 gm.). The hybrid for this leukemia was initially introduced by Dr. George E. Jay because of the correspondence of histocompatibility genes in the parent strains. The mice were maintained at laboratory temperatures of 72 to 78° F. in plastic cages and offered Purina chow and water *ad libitum*.

Tumor transplantation was conducted under aseptic conditions. A uniform saline suspension of spleen cells was prepared from stock DBA mice with advanced leukemia. The spleens were removed on the 7th to 9th day following subcutaneous tumor implantation when the local tumor at the site of leukemic inoculation was approximately 10 mm. in diameter. At this time the spleens weighed 500 to 750 mg., which is about 5 times the weight of the normal spleen. Each spleen was introduced into a 1 cc. tuberculin syringe and passed back and forth through a 20-gauge needle into 60 ml. of normal saline. The cell suspension was filtered through sterile cotton gauze. The number of cells in the resulting cell suspension was estimated by counting in a hemocytometer. The suspension usually contained 5 to 20 million cells per ml.

The hybrid mice were inoculated subcutaneously (on day 0) in the right hind leg with 0.1 ml. of the leukemic cell suspension. The concentration of inoculum received by each mouse (*ca.* 500,000 to 2,000,000 cells) was usually about 10 to 100 times greater than that required to give 100 percent tumor takes and death when a serial dilution of the inoculum was injected into control hybrid mice.

Treatment was initiated at 7 to 10 days when the local tumor at the site of leukemic inoculation measured 8 to 12 mm. in diameter. At this time the disease is systemic as well as local and it is readily transmissible by transplantation of blood, spleen, or other organs (*12*, *14*, *16*). In most of the experiments in the current study, when treatment was initiated, mice were selected at random for sacrifice, and the blood and/or spleen were transplanted to other hybrid mice. In all cases the transplants were successful and the animals succumbed.

The mice were assigned at random to the various injection groups. In no instance, however, were any mice put into the experiment which did not show local tumors of 8 to 12 mm. diameter.

Table 1 lists the compounds employed, the source, references (*4*, *17–34*), the vehicle, and the experiment numbers in which the compounds were used. All treatments were subcutaneous in the axillary region. In those instances in which more than one drug was employed, the individual drugs were injected in opposite axillas. The drugs were ordinarily injected at 0.01 ml. per gm. of body weight of mouse.

In most experiments treatment was given daily until all the animals succumbed. With amethopterin such a schedule had been found optimal for advanced leukemia (*12*). In several experiments treatment was discontinued after the median survival time of a group was established.

TABLE 1.—*List of compounds*

Compound No.	Compound	Source	Reference	Vehicle	Experiment No.
1	Methyl-bis(β-chloroethyl)amine hydrochloride (Nitrogen mustard HN_2, Mustargen, Merck).	Sharp & Dohme	(4)*	0.85% Saline	J294
2	Amethopterin (4-amino-N^{10}-methylpteroylglutamic acid, Methotrexate).	Calco; Lederle Laboratories.	(4)	2% $NaHCO_3$	All experiments
3	Hydrocortisone (Compound F)	Merck & Co., Inc.	(4)	1% CMC†	J299
4	*N*-Methylformamide	Rohm and Haas Co.	(4)	0.85% Saline	J299, 317
5	*N*-Methylacetamide	Rohm and Haas Co.	(4)	0.85% Saline	J299
6	6-Mercaptopurine (Purinethol)	Burroughs Wellcome & Co. (U. S. A.) Inc.	(4)	Dilute NaOH	J280, J281
7	Urethan	Eli Lilly and Company	(4)	2% $NaHCO_3$	J313
9	Chloramphenicol (Chloromycetin)	Parke, Davis & Company	(4)	1% CMC	J313
11	8-Azaguanine (5-amino-7-hydroxy-1*H*-triazolo[*d*]-pyrimidine, Guanazolo).	Calco; Lederle Laboratories.	(4)	Dilute NaOH	J280
12	8-Azaxanthine	Lederle Laboratories Division, American Cyanamid Company.	(4)	Dilute NaOH	J313
13	Benzimidazole	Eastman Kodak Company	(4)	Dilute NaOH	J313
14	D-Glucosamine	Eastman Kodak Company	(4)	Water	J313
15	2,6-Diaminopurine	Burroughs Wellcome & Co. (U. S. A.) Inc.	(4)	1% CMC	J299, 313, 317
17	Diethylstilbestrol (stilbestrol)	Specific Pharmaceuticals, Inc.	(4)	1% CMC	J313
18	2, 4-Diamino-5-(3′, 4′-dichlorophenyl)-6-ethylpyrimidine.	Burroughs Wellcome & Co. (U. S. A.) Inc.	(4)	1% CMC	J298, 299, 313, 317
19	2,4-Diamino-5-(*p*-chlorophenyl)-6-ethylpyrimidine (Daraprim).	Burroughs Wellcome & Co. (U. S. A.) Inc.	(4)	1% CMC	J315, 317
20	1,5-Diaminobiuret	Southern Research Institute.	(4)	Water	J313
21	DL-Ethionine	Merck & Co., Inc.	(4)	1% CMC	J316
22	β-2-Thienylalanine	Florida State University	(4)	1% CMC	J316
23	Deoxypyridoxine hydrochloride	Merck & Co., Inc.	(4)	1% CMC	J316
24	Colchicine	John Wood	(4)	Water	J294
26	5-Methoxytoluquinone	Merck & Co., Inc.	(4)	1% CMC	J317
28	Azaserine (O-diazoacetyl-L-serine)	Parke, Davis & Company	(4)	Water	J280, 281
	Thioguanine	Burroughs Wellcome & Co. (U. S. A.) Inc.	(17)	Dilute NaOH	J280, 281

Aminopterin	Calco; Lederle Laboratories.	(*18*, *19*)	2% $NaHCO_3$	J294
4,6-Diamino-1-(4′-chlorophenyl)-1,2-dihydro-2,2-dimethyl-*s*-triazine hydrochloride (D20).	Childrens Cancer Research Foundation, Boston.	(*20*, *21*)	Water	J315
4,6-Diamino-1-(3′,4′-dichlorophenyl)-1,2-dihydro-2,2-dimethyl-*s*-triazine hydrochloride (D54).	Childrens Cancer Research Foundation, Boston.	(*20*, *21*)	Water	J315, 316
4-Aminopyrazolo[3,4-*d*]pyrimidine sulfate	R. K. Robins; Ti Li Loo	(*22*, *23*)	Water	J284
Reserpine (Serpasil)	Ciba Pharmaceutical Products Inc.	(*24*)	Water	J284
5-Fluorouracil	Hoffman-LaRoche Inc.	(*25*)	Saline	J319
2-Ethylamino-1,3,4-thiadiazole hydrochloride	Lederle Laboratories Division, American Cyanamid Company.	(*26*, *27*)	2% $NaHCO_3$	J310, 319
2-Amino-1,3,4-thiadiazole	Lederle Laboratories Division, American Cyanamid Company.	(*26*, *27*)	2% $NaHCO_3$	J311
6-Azauracil	E. R. Squibb & Sons	(*28*, *29*)	Water	S159
Potassium oxamate	S. Colowick	(*30*)	Water	S159
Ribonuclease (crystalline pancreatic)	The Armour Laboratories	(*31*)	Water	S159
Puromycin · 2HCl	American Cyanamid Company.	(*32*)	Water	J310, 311
Bryonia starch (plant extract)	A. Perrault	(*33*)	Water	J310
3-Oxocyclopentanecarboxylic acid	A. Schrecker	(*34*)	2% $NaHCO_3$	J311
Nicotinamide	Eastman Kodak Company	(*26*, *27*)	Water	J319

*The numbered compounds were employed in the Gellhorn-Hirschberg report (*4*). Compounds #3, 4, 5, 7, 9, 12, 13, 14, 15, 17, 18, 20, 22, 26 were kindly provided by Dr. Erich Hirschberg. A selected bibliography for these compounds is included in the report (*4*). The additional compounds (*17* to *34*) were selected because of already demonstrated or for possible antileukemic activity.

†CMC = carboxymethylcellulose.

Observations were made of body weight, size of the local tumor, and survival time.

In each experiment, the drugs were usually administered in a series of constant dose levels estimated from preliminary data to range from doses giving marked toxicity down to nontoxic doses. The doses for each drug were spaced logarithmically over the intervals listed in table 2, the ratio of successive doses ranging generally from 0.5 to 0.7. Typically, each group contained 10 animals.

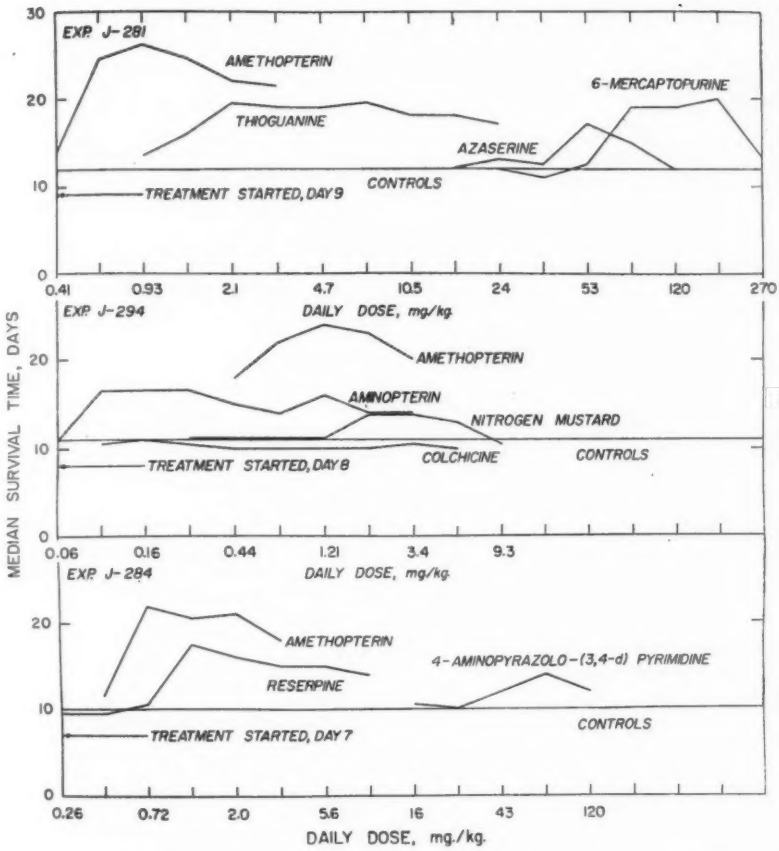
For an active compound, as the dose is increased, the survival time increases. When host toxicity becomes limiting, with further increase in dosage, the survival time diminishes. The optimal dose can be considered to be the dose at which the animals show the maximum increase in survival time.

Results

The data of 14 experiments are summarized in tables 2 and 3. Data of several typical experiments are summarized graphically in text-figure 1, including the median survival times achieved at the various dosages for each compound.

In each experiment amethopterin was employed as a standard. Of the 38 drugs tested, including 23 of those employed in the Gellhorn-Hirschberg study (*4*) and 15 additional drugs, amethopterin was clearly the most effective compound. It consistently gave the greatest increase in survival time of the leukemic mice. The median survival times for amethopterin, observed at the optimal daily doses, in the 14 experiments (range, 20 to 26.5 days) were 2.0 to 2.9 times greater than the median survival times of the untreated controls (range, 9 to 12 days). In terms of the median remaining lifetime of the mice from the time of initiation of treatment, there was a 4.6- to 16.5-fold increase—the median remaining lifetime of controls ranged from 1 to 4 days and this was increased to from 12 to 19.5 days at optimal doses in animals treated with amethopterin.

The relative increases in median survival times observed at optimal doses for the various compounds, as compared with the amethopterin standard, are listed in the last column of table 2 and are arranged in order of effectiveness in table 3. The tranquilizing drug reserpine was 62 percent as effective as amethopterin. The purine antagonists, 6-mercaptopurine, thioguanine, and 8-azaguanine were approximately equally effective and from 54 to 57 percent as effective as amethopterin in increasing the lifespan of the leukemic mice. The pyrimidine antagonist 5-fluorouracil was 52 percent as effective as amethopterin. Aminopterin (42%) and the 2 thiadiazole derivatives, 2-amino-1,3,4-thiadiazole and 2-ethylamino-1,3,4-thiadiazole (46% and 37 to 38% of amethopterin, respectively) were next in relative effectiveness. The glutamine antagonist azaserine showed similar activity (36 to 39%). Both of the diaminopyrimidines and the pyrazolo pyrimidine also showed definite antileukemic activity (26 to 50%). A lesser order of activity (14 to 29% of



TEXT-FIGURE 1.—Illustrations of the use of the assay system employing advanced leukemia (L1210) for comparing the efficacies of various agents. Each agent is tested in a series of dose levels so as to determine its maximal effectiveness in increasing survival time of the tumor-bearing host. The maximal survival time attained provides an index of effectiveness with no regard given to the dose level required. These 3 experiments are summarized in table 2.

amethopterin) was exhibited by nitrogen mustard (HN_2), *N*-methylformamide, 1-5 diaminobiuret, and D54. The other compounds employed showed little or no activity.

Therapeutic synergism was not observed with thioguanine plus azaserine or 6-mercaptopurine plus azaserine, table 2 (*35*).[3] Nicotinamide antagonized the antileukemic action of the thiadiazole derivative (table 2).

The current results on treatment of advanced leukemia are compared with the results of the previous study (*4*) where treatment was initiated

[3] The range was not covered adequately for the combination of 6-mercaptopurine plus azaserine. In a subsequent experiment where the dose range was more extensive for several schedules of treatment, evidence for therapeutic synergism was obtained.

TABLE 2.—*Treatment of advanced leukemia L1210 in mice: summary of 14 experiments*

Experiment No.	Compound No.	Compound	Dose range, daily (*mg./kg.*)	Optimal dose level, daily (*mg./kg.*)	Median survival time at optimal dose (*days*)	Relative median survival time. Untreated controls equal 1	Median remaining lifetime. Day of treatment equals 0 (*days*)	Relative median remaining lifetime. Untreated controls equal 1	Increase in median survival time over controls (*days*)	Relative increase in median survival time. Amethopterin increase equals 1
J280 daily from day 8	2	Amethopterin	0. 27–4. 7	0. 93	24	2. 4	16	8. 0	14	1. 00
	6	6-Mercaptopurine	23. 7–180	180	18	1. 8	10	5. 0	8	0. 57
	11	8-Azaguanine	10. 5–120	53. 3	18	1. 8	10	5. 0	8	0. 57
	28	Azaserine	7–120	53. 3	15. 5	1. 6	7. 5	3. 8	5. 5	0. 39
		Thioguanine	3. 1–53. 3*	3. 1	18	1. 8	10	5. 0	8	0. 57
		Controls	—	—	10	1. 0	2	1. 0	—	—
J281 daily from day 9	2	Amethopterin	0. 41–3. 1	0. 93	26	2. 2	17	5. 7	14	1. 00
	6	6-Mercaptopurine	23. 7 –270	180	20	1. 7	11	3. 7	8	0. 57
	28	Azaserine	15. 8 –120	53. 3	17	1. 4	8	2. 7	5	0. 36
		Thioguanine	0. 93–23. 7	7	19. 5	1. 6	10. 5	3. 5	7. 5	0. 54
		Azaserine + 6-Mercaptopurine	10. 5+23. 7–80+180*	10. 5+23. 7	20	1. 7	11	3. 7	8	0. 57
		Azaserine + Thioguanine	10. 5+0. 62–80+4. 7	15. 8+0. 93	19. 5	1. 6	10. 5	3. 5	7. 5	0. 54
		Controls	—	—	12	1. 0	3. 0	1. 0	—	—
J284 daily from day 7	2	Amethopterin	0. 43–3. 35	0. 72	22	2. 2	15	5. 0	12	1. 00
		4-Aminopyrazolo[3,4-*d*]pyrimidine sulfate	15. 5 –120	72	14	1. 4	7	2. 3	4	0. 33
		Reserpine	0. 26–9. 3	1. 2	17. 5	1. 8	10. 5	3. 5	7. 5	0. 62
		Controls	—	—	10	1. 0	3	1. 0	—	—
J294 daily from day 8	1	Nitrogen mustard	0. 26–9. 3	2. 01	14	1. 3	6	2. 0	3	0. 23
	2	Amethopterin	0. 44–3. 35	1. 21	24	2. 2	16	5. 3	13. 0	1. 00
	24	Colchicine	0. 1 –5. 58	0. 16	11	1. 0	3	1. 0	0	0
		Aminopterin	0. 06–3. 35	0. 16	16. 5	1. 5	8. 5	2. 8	5. 5	0. 42
		Controls	—	—	11	1. 0	3	1. 0	—	—

J298 daily from day 10	2	Amethopterin	0. 45–3. 5	0. 75	26. 5	2. 4	16. 5	16. 5	15. 5	1. 00
	18	2,4-Diamino-5-(3,4-dichlorophenyl)-6-ethylpyrimidine	2. 09–45	45	15	1. 4	5	5. 0	4	0. 26
		Controls	—	—	11	1. 0	1	1. 0	—	—
J299 daily from day 9	2	Amethopterin	0. 46–2. 1	0. 76	26	2. 2	17	5. 7	14	1. 00
	3	Hydrocortisone	5. 83–75†	27	12‡	1. 0	3	1. 0	0	0
	4	*N*-Methylformamide	27–347	347	14	1. 2	5	1. 7	2	0. 14
	5	*N*-Methylacetamide	75–963†	75–963	12	1. 0	3	1. 0	0	0
	15	2,6-Diaminopurine	5. 83–75†	5. 83–75	12	1. 0	3	1. 0	0	0
	18	2,4-Diamino-5-(3′,4′-dichlorophenyl)-6-ethylpyrimidine	9. 72–125	9. 72	17. 5	1. 5	8. 5	2. 8	5. 5	0. 39
		Controls	—	—	12	1. 0	3	1. 0	—	—
J310 daily from day 8	2	Amethopterin	0. 47–3. 6	1. 3	26	2. 3	18	5. 1	14. 5	1. 00
		Puromycin. 2HCl	125–500	250	13	1. 1	5	1. 4	1. 5	0. 10
		Bryonia starch	125–500†	250–500	12	1. 0	4	1. 1	0. 5	0. 03
		2-Ethylamino-1,3,4-thiadiazole hydrochloride	125–1000	250	17	1. 5	9	2. 6	5. 5	0. 38
		Controls	—	—	11. 5	1. 0	3. 5	1. 0	—	—
J311 from day 7	2	Amethopterin	0. 38–6	0. 75	26. 5	2. 9	19. 5	9. 8	17. 5	1. 00
		Puromycin. 2HCl (daily)	250–500*	250	11	1. 2	4	2	2	0. 12
		Puromycin. 2HCl (every 2 days)	250–500*	250–500	10	1. 1	3	1. 5	1	0. 06
		Puromycin. 2HCl (every 3 days)	250–500*	250	11	1. 2	4	2	2	0. 12
		Puromycin. 2HCl (every 4 days)	250–500*	250	11	1. 2	4	2	2	0. 12
		2-Amino-1,3,4-thiadiazole	62. 5–500	125	17	1. 9	10	5	8	0. 46
		3-Oxocyclopentanecarboxylic acid	15. 5–1000†	62. 5	10	1. 1	3	1. 5	1	0. 06
		Control	—	—	9	1. 0	2	1. 0	—	—

TABLE 2.—*Treatment of advanced leukemia L1210 in mice: summary of 14 experiments*—Continued

Experiment No.	Compound No.	Compound	Dose range, daily (*mg./kg.*)	Optimal dose level, daily (*mg./kg.*)	Median survival time at optimal dose (*days*)	Relative median survival time. Untreated controls equal 1	Median remaining lifetime. Day of treatment equals 0 (*days*)	Relative median remaining lifetime. Untreated controls equal 1	Increase in median survival time over controls (*days*)	Relative increase in median survival time. Amethopterin increase equals 1
J313 daily from day 8	2	Amethopterin	0. 56–2. 34	1. 17	20	2. 0	12	6. 0	10	1. 00
	7	Urethan	150–600†	150–600	10. 5	1. 0	2. 5	1. 2	0. 5	0. 05
	9	Chloramphenicol	150–600†	600	11	1. 1	3	1. 5	1	0. 10
	12	8-Azaxanthine	150–600†	150	11	1. 1	3	1. 5	1	0. 10
	13	Benzimidazole	150–600†	150–600	10	1. 0	2	1. 0	0	0
	14	D-Glucosamine	1000†	1000	10	1. 0	2	1. 0	0	0
	15	2,6-Diaminopurine	150–300†	300	11	1. 1	3	1. 5	1	0. 10
	17	Diethylstilbestrol	9. 4–75†	9. 4–75	10	1. 0	2	1. 0	0	0
	18	2,4-Diamino-5-(3,4-dichlorophenyl)-6-ethylpyrimidine	4. 7–75	4. 7–37. 5	15	1. 5	7	3. 5	5	0. 50
	20	1,5-Diaminobiuret	75–300	300	12	1. 2	4	2. 0	2	0. 20
		Controls	—	—	10	1. 0	2	1. 0	—	—
J315 daily from day 7	2	Amethopterin	0. 65–1. 9*	0. 65	26. 5	2. 6	19. 5	6. 5	16. 5	1. 00
	19	2,4-Diamino-5-(4′-chlorophenyl)-6-ethylpyrimidine (Daraprim).	171. 5–500	245	16	1. 6	9	3. 0	6	0. 36
		4,6-Diamino-1-(*p*-chlorophenyl)-1,2-dihydro-2,2-dimethyl-*s*-triazine hydrochloride (D20).	120–350	120	10	1. 0	3	1. 0	0	0
		4,6-Diamino-1-(3′,4′-dichlorophenyl)-1,2-dihydro-2,2-dimethyl-*s*-triazine hydrochloride (D54).	120–350*	120	13	1. 3	6	2. 0	3	0. 18
		Controls	—	—	10	1. 0	3	1. 0	—	—
	2	Amethopterin	0. 39–1. 8	0. 39	24	2. 4	17	5. 7	14	1. 00

	21	DL-Ethionine	38. 9–300†	38. 9	10	1. 0	3	1. 0	0	0
	22	β-2-Thienylalanine	38. 9–180†	38. 9–180	10	1. 0	3	1. 0	0	0
J316 daily from day 7	23	Deoxypyridoxine hydrochloride.	23. 3–180†	23. 3–180	10	1. 0	3	1. 0	0	0
		4,6-Diamino-1-(3′,4′-dichlorophenyl)-1,2-dihydro-2,2-dimethyl-s-triazine hydrochloride (D54).	23. 3–180	38. 9	14	1. 4	7	2. 3	4	0. 29
		Controls	—	—	10	1. 0	3	1. 0	—	—
	2	Amethopterin	0. 47–3. 6	0. 78	24	2. 7	17	8. 5	15	1. 00
	4	N-Methylformamide	78–600	360	13	1. 4	6	3. 0	4	0. 27
	15	2,6-Diaminopurine	130–600†	600	11	1. 2	4	2. 0	2	0. 13
J317 daily from day 7	18	2,4-Diamino-5-(3′,4′-dichlorophenyl)-6-ethylpyrimidine.	2. 16–78	47	14	1. 6	7	3. 5	5	0. 33
	19	2,4-Diamino-5-(4′-chlorophenyl)-6-ethylpyrimidine (Daraprim).	78–600	216	13	1. 4	6	3. 0	4	0. 27
	26	5-Methoxytoluquinone	17–130†	47	10	1. 1	3	1. 5	1	0. 07
		Controls	—	—	9	1. 0	2	1. 0	—	—
	2	Amethopterin	0. 37–2. 44	1. 45	23. 5	2. 4	16. 5	5. 5	13. 5	1. 00
		5-Fluorouracil	18. 7–400	31. 1	17	1. 7	10	3. 3	7	0. 52
J319 daily from day 7		2-Ethylamino-1,3,4-thiadiazole hydrochloride.	86. 4–400†	240–400	15	1. 5	8	2. 7	5	0. 37
		2-Ethylamino-1,3,4-thiadiazole hydrochloride + nicotinamide. (500 mg./kg.).	86. 4–667†	667	10	1. 0	3	1. 0	0	0
		Controls	—	—	10	1. 0	3	1. 0	—	—
	2	Amethopterin	0. 62–1. 4*	0. 62	25. 5	2. 3	18. 5	4. 6	14. 5	1. 00
S159 daily from day 7		6-Azauracil	71. 5–715†	429–715	12	1. 1	5	1. 2	1	0. 07
		Potassium oxamate	250–1000†	250–1000	11	1. 0	4	1. 0	0	0
		Ribonuclease	250–1000†	250–1000	11	1. 0	4	1. 0	0	0
		Controls	—	—	11	1. 0	4	1. 0	—	—

* and † are used to indicate those experiments in which the possibility remains open that the optimal dose level is outside the dose range employed. Where all dose levels showed toxicity, denoted by *, the optimal level may be lower than any employed. In experiments denoted by † no clear toxicity appeared at any dose level tested and the optimal level may be higher than any employed. In some experiments, although the maximum median survival time occurred at one of the extreme dose levels tested, the data indicated no advantage in testing at still more extreme levels. These experiments are not noted.

‡With hydrocortisone no increase in the median survival time occurred at any of the dose levels tested. The average survival time, however, showed a definite peak at one of the intermediate levels.

TABLE 3.—*Relative effectiveness of compounds in increasing survival time of mice with leukemia L1210*

Compound No.	Compound	Advanced leukemia. Relative increase in median survival time (*amethopterin*=100%)	Early leukemia. Rating based on percent increase in average survival time beyond controls*
2	Amethopterin	100	+
	Reserpine	62	
11	8-Azaguanine	57	+
6	6-Mercaptopurine	57, 57	+
	Thioguanine	54, 57	
	5-Fluorouracil	52	
	2-Amino-1,3,4-thiadiazole	46	
	Aminopterin	42	
	2-Ethylamino-1,3,4-thiadiazole hydrochloride	37, 38	
28	Azaserine	36, 39	±
18	2,4-Diamino-5-(3′,4′-dichlorophenyl)-6-ethylpyrimidine	26, 33, 39, 50	−
	4-Aminopyrazolo[3,4-*d*]pyrimidine sulfate	33	
19	2,4-Diamino-5-(4′-chlorophenyl)-6-ethylpyrimidine	27, 36	±
	4,6-Diamino-1-(3′,4′-dichlorophenyl)-1,2-dihydro-2,2-dimethyl-*s*-triazine hydrochloride (D54)	18, 29	
1	Nitrogen mustard	23	±
4	*N*-Methylformamide	14, 27	+
20	1,5-Diaminobiuret	20	±
	Puromycin·2HCl	10, 12	
9	Chloramphenicol	10	−
12	8-Azaxanthine	10	−
15	2,6-Diaminopurine	0, 10, 13	−
26	5-Methoxytoluquinone	7	−
	6-Azauracil	7	
	3-Oxocyclopentanecarboxylic acid	6	
7	Urethan	5	−
	Bryonia starch	3	
3	Hydrocortisone	0	−
5	*N*-Methylacetamide	0	−
13	Benzimidazole	0	−
14	D-Glucosamine	0	−
17	Diethylstilbestrol	0	−
21	DL-Ethionine	0	−
22	β-2-Thienylalanine	0	−
23	Deoxypyridoxine hydrochloride	0	−
24	Colchicine	0	−
	4,6-Diamino-1-(4′-chlorophenyl)-1,2-dihydro-2,2-dimethyl-*s*-triazine hydrochloride (D20)	0	
	Potassium oxamate	0	
	Ribonuclease	0	

*From data of Skipper and Thomson (*see* reference 4, page 44); − =failure to provide an increase in life span of at least 20 percent; ± =increase in life span of 20 to 50 percent; + =increase in life span of 50 to 150 percent.

early in the course of the disease (table 3). In the study with early leukemia, the compounds were assigned ratings based on the percent increase in survival time beyond controls. The close correlation for most of the compounds employed may be noted (table 3, cols. 3 and 4). In general, the compounds that received the highest ratings in the current study were also the most active ones in the previous study employing

early leukemia. The only two differences noted were with respect to *N*-methylformamide and 2,4-diamino-5-(3′,4′-dichlorophenyl)-6-ethylpyrimidine. The *N*-methylformamide received a lower rating in the current study. The 3′,4′-dichlorophenyl pyrimidine, rated as negative against early leukemia, showed definite activity against advanced leukemia.

Discussion

The current experiments illustrate the use of an antitumor-assay procedure employing advanced leukemia in mice. The procedure is readily adaptable as a screening tool and may be employed in the quantitative evaluation of the relative effectiveness of drugs or treatments.

The advantages that may be obtained with a system such as the one employed here, in which treatment is initiated late in the course of the disease, may be outlined with respect to several categories of interest.

Discrimination Between Active and Inactive Compounds

In this system, a positive compound is one that gives a significant and reproducible increase in survival time of the animals as compared with controls. The current data plus previous studies (*12*, *14*) show that the system affords good discrimination between effective and ineffective compounds. Within any particular experiment the compounds can be ordered in terms of effectiveness, and when a standard such as amethopterin is employed, compounds in a series of experiments can be rated with respect to the standard. In this connection it may be noted that in the current study various compounds showed definite antileukemic activity. However, when reference was made in each experiment to amethopterin as a standard, on a quantitative basis, none of the compounds was as much as 60 percent as effective as the standard. In other studies (*36*) several congeners of amethopterin were as effective as, or more effective than, amethopterin.

False positives.—This system would tend to minimize the chances of improperly evaluating an inactive compound as positive.

1) The system is sufficiently sensitive so that the compounds may be rated not only as positive or negative but also with respect to degree of effect. Since a range of dosage is employed, the adjacent dose-responses serve as checks on each other.

2) Advanced leukemia in mice is less sensitive to treatment than early leukemia (*7*, *12*). The system will therefore tend to select as positive, compounds with definite activity.

3) Treatment initiated against early leukemia, at a time when the tumor cells are localized, does not distinguish between inhibition of the "take process" and inhibition of tumor growth. This complication is eliminated in studies with advanced leukemia, because, in addition to the presence of an established tumor at the site of leukemic inoculation, the disease is systemic at the time that treatment is initiated.

4) Reduction in food intake resulting from nonspecific drug toxicity may retard tumor growth and increase the survival time of animals with early leukemia (*37*). The current system would tend to eliminate such false positives. Although food restriction initiated late in the course of the disease may diminish the size of the local tumor at the site of leukemic inoculation, no degree of food or water restriction has been capable of increasing the survival time of mice with advanced leukemia (*37*).

False negatives.—There is little chance that a compound will be classified as negative if it is capable of important antileukemic action in the system. Utilization of a whole series of dose levels ranging from nontoxic doses to quite toxic doses ensures observation of potential antileukemic action. However, since it is more difficult to increase the survival time of mice with advanced leukemia, as compared with early leukemia, it is conceivable that a compound which exerted very weak effect could be classified as negative. This, of course, could be desirable where a standard with considerable activity is on hand.

If the appropriate dose range were not employed, or if the doses were too widely spaced, a positive compound could be missed. These possibilities become minimal when adequate toxicity data is available and could usually be recognized as having occurred because of the nature of the testing. For example, if the dose range employed were ineffective in increasing survival time or inhibiting the growth of the local tumor at the site of leukemic inoculation and there were no accompanying weight loss or other symptoms of toxicity, the dose range could be increased in a repeat experiment. If all doses employed were clearly too toxic, the dose range could be reduced. If there were too sharp a change from nontoxic to toxic doses, the dose interval could be narrowed. Size of the local tumor and body-weight changes could serve as guides in such judgments.

There is a special case in which treatment initiated late in the course of the disease could more readily detect a positive compound than treatment initiated early. If a compound had antitumor action, which occurred only at doses that had definite toxicity for the host, treatment initiated early could fail to increase the survival time of the mice. Under these circumstances, retardation of growth of a local tumor might be detected but it would be necessary to rule out any possible role of nonspecific drug toxicity for the host. On the other hand, with treatment initiated late in the course of the disease, diminished tumor sizes and increases in survival time could be evident even though the animals succumbed ultimately to drug toxicity.

Economy of the System

Several economies with respect to the time and number of manipulations required for testing are inherent in the current system.

1) Since treatment is initiated only a few days prior to death, the investment in injection effort for inactive compounds and for control groups is reduced.

2) For compounds that have antileukemic activity there may also be a saving in time and manipulation required during the period of treatment following the death of control mice. This results from the ordinarily lesser effectiveness of drugs in prolonging survival time when the initiation of treatment is delayed. For example, while median survival times on the order of 50 to 70 days have been attained in the treatment of early leukemia with amethopterin (*13*), the optimal treatment of advanced leukemia yields median survival times on the order of only 20 to 30 days. The increased difficulty in treating mice with advanced leukemia may be traceable to the increased numbers of leukemic cells and the inability of the mice to tolerate as much drug (*11*, *12*).

3) For early leukemia, it is necessary to determine in additional studies whether demonstrated activity of a compound is attributable merely to a reduction in caloric intake resulting from nonspecific drug toxicity. This is not necessary for advanced leukemia since, as indicated, demonstrated activity is not attributable to reduction in caloric intake (*37*).

Other Investigations

The assay system with advanced leukemia may be employed in detailed investigation of individual compounds of interest. It may be used to determine the influence of treatment schedule for a drug, including determination of optimal doses, optimal intervals between treatment, optimal duration of treatment, etc. (*12*, *36*). Although none of the compounds employed in the current study was as effective as amethopterin, conceivably, if employed on other schedules of treatment, the antileukemic action could have been increased (*36*). The assay may be employed in studies of both synergism and antagonism (*16*, *35*) and in detailed investigation of the influence of drugs on the host-tumor relationship (*36*, *37*).

Since treatment of advanced tumor in mice may more closely approximate a clinical situation, it is suggested that assay procedures such as the one presented here, not only with leukemia but with solid tumors as well, may prove to be valuable tools in cancer chemotherapy.

References

(*1*) Chemical target: Cancer. Chem. & Eng. News 33: 5138–5142, 1955.

(*2*) Stock, C. C.: Studies in experimental cancer chemotherapy. Current Res. in Cancer Chemother., Rep. No. 3–55: 3–19, 1955.

(*3*) ———: Experimental cancer chemotherapy. Advances in Cancer Res. (Greenstein, J. P., and Haddow, A., eds.). New York, Academic Press, Inc., 1954, vol. 2, pp. 425–429.

(*4*) Gellhorn, A., and Hirschberg, E. (eds.): Investigation of diverse systems for cancer chemotherapy screening. Cancer Res. Supp. No. 3: 1955, 125 pp.

(*5*) Goldin, A., Mantel, N., Greenhouse, S. W., Venditti, J. M., and Humphreys, S. R.: Estimation of the antileukemic potency of the antimetabolite aminopterin, administed alone and in combination with citrovorum factor or folic acid. Cancer Res. 13: 843–850, 1953.

(*6*) ———: Effect of delayed administration of citrovorum factor on the antileukemic effectiveness of aminopterin in mice. Cancer Res. 14: 43–48, 1954.

(7) ———: Factors influencing the specificity of action of an antileukemic agent (aminopterin). Time of treatment and dosage schedule. Cancer Res. 14: 311–314, 1954.

(8) GOLDIN, A., VENDITTI, J. M., HUMPHREYS, S. R., DENNIS, D., MANTEL, N., and GREENHOUSE, S. W.: Factors influencing the specificity of an antileukemic agent (aminopterin). Multiple treatment schedules plus delayed administration of citrovorum factor. Cancer Res. 15: 57–61, 1955.

(9) ———: A quantitative comparison of the antileukemic effectiveness of two folic acid antagonists. J. Nat. Cancer Inst. 15: 1657–1664, 1955.

(10) GOLDIN, A., VENDITTI, J. M., HUMPHREYS, S. R., DENNIS, D., and MANTEL, N.: Factors influencing the specificity of action of an antileukemic agent (aminopterin): Host age and weight. J. Nat. Cancer Inst. 16: 709–721, 1955.

(11) GOLDIN, A., VENDITTI, J. M., HUMPHREYS, S. R., and MANTEL, N.: The influence of the concentration of leukemic inoculum on the effectiveness of treatment. Science 123: 840, 1956.

(12) ———: Modification of treatment schedules in the management of advanced mouse leukemia with amethopterin. J. Nat. Cancer Inst. 17: 203–212, 1956.

(13) GOLDIN, A., VENDITTI, J. M., HUMPHREYS, S. R., DENNIS, D., and MANTEL, N.: Studies on the management of mouse leukemia (L1210) with antagonists of folic acid. Cancer Res. 15: 742–747, 1955.

(14) HUMPHREYS, S. R., VENDITTI, J. M., MANTEL, N., and GOLDIN, A.: Studies with advanced leukemia (L1210) in mice. (Abstract.) Proc. Am. Assoc. Cancer Res. 2: 215–216, 1957.

(15) LAW, L. W., DUNN, T. B., BOYLE, P. J., and MILLER, J. H.: Observations on the effect of a folic-acid antagonist on transplantable lymphoid leukemias in mice. J. Nat. Cancer Inst. 10: 179–192, 1949.

(16) VENDITTI, J. M., HUMPHREYS, S. R., MANTEL, N., and GOLDIN, A.: Combined treatment of advanced leukemia (L1210) in mice with amethopterin and 6-mercaptopurine. J. Nat. Cancer Inst. 17: 631–638, 1956.

(17) CLARKE, D. A., PHILIPS, F. S., STERNBERG, S. S., STOCK, C. C., and HITCHINGS, G. H.: Inhibition of sarcoma 180 by thioguanine, an analog of 6-mercatopurine. (Abstract.) Proc. Am. Assoc. Cancer Res. 1: 9, 1954.

(18) SEEGER, D. R., SMITH, J. M., JR., and HULTQUIST, M. E.: Antagonist for pteroylglutamic acid. J. Am. Chem. Soc. 69: 2567, 1947.

(19) FARBER, S., DIAMOND, L. K., MERCER, R. D., SYLVESTER, R. F., JR., and WOLFF, J. A.: Temporary remissions in acute leukemia in children produced by folic acid antagonist, 4-aminopteroylglutamic acid (aminopterin). New England J. Med. 238: 787–793, 1948.

(20) MODEST, E. J., FOLEY, G. E., PECHET, M. M., and FARBER, S.: A series of new, biological significant dihydrotriazines. J. Am. Chem. Soc. 74: 855–856, 1952.

(21) MODEST, E., FARBER, S., and FOLEY, G. E.: Structure–Biological activity relationships in a series of biologically active dihydrotriazines. (Abstract.) Proc. Am. Assoc. Cancer Res. 1: 33, 1954.

(22) SKIPPER, H. E., ROBINS, R. K., and THOMSON, J. R.: Inhibition of experimental neoplasms by 4-aminopyrazolo (3,4-*d*) pyrimidine. Proc. Soc. Exper. Biol. & Med. 89: 594–596, 1955.

(23) SKIPPER, H. E., ROBINS, R. K., THOMSON, J. R., BROCKMAN, R. W., and SCHABEL, F. M., JR.: Further observations on the inhibition of experimental neoplasms by pyrazolo (3,4-*d*) pyrimidines. (Abstract.) Proc. Am. Assoc. Cancer Res. 2: 147, 1956.

(24) GOLDIN, A., BURTON, R. M., HUMPHREYS, S. R., and VENDITTI, J. M.: Antileukemic action of reserpine. Science 125: 156–157, 1957.

(25) HEIDELBERGER, C., MOOREN, D., GRIESBACH, L., MONTAG, B. J., DUSCHINSKY, R., PLEVEN, E., and SCHNITZER, R.: Fluorinated pyrimidines, a new class of tumor-inhibitory compounds. (Abstract.) Proc. Am. Assoc. Cancer Res. 2: 212, 1957.

(*26*) Oleson, J. J., Sloboda, A., Troy, W. P., Halliday, S. L., Landes, M. J., Angier, R. B., Semb, J., Cyr, K., and Williams, J. H.: The carcinostatic activity of some 2-amino-1,3,4-thiadiazoles. J. Am. Chem. Soc. 77: 6713–6714, 1955.

(*27*) Shapiro, D. M., Shils, M. E., Fugmann, R. A., and Friedland, I. M.: Quantitative biochemical differences between tumor and host as a basis for cancer chemotherapy IV. Niacin and 2 ethylamino-1,3,4-thiadiazole. Cancer Res. 17: 29–33, 1957.

(*28*) Hakala, M. T., Law, L. W., and Welch, A. D.: Inhibitory activity of 6-azauracil, 6-uracil methyl sulfone, and related compounds on the growth of mouse lymphomas and sarcoma 180. (Abstract.) Proc. Am. Assoc. Cancer Res. 2: 113, 1956.

(*29*) Barlow, R. B., and Welch, A. D.: A synthesis of "6-azauracil" (1,2,4-triazine-3,5 (2*H*, 4*H*)-dione), an analog of uracil. J. Am. Chem. Soc., 78: 1258–1259, 1956.

(*30*) Papaconstantinou, J., and Colowick, S. P.: Effects of oxamic acid on metabolism of ascites tumors. Federation Proc. 16: 230–231, 1957.

(*31*) Ledoux, L.: Action of ribonuclease on two solid tumours *in vivo*. Nature, London 176: 36–37, 1955.

(*32*) Troy, W., Smith, S., Personeus, G., Moser, L., James, E., Sparks, S. J., Stevens, M., Halliday, S., McKenzie, D., and Oleson, J. J.: The effect of puromycin on experimental tumors. Antibiotics Ann. 186–190, 1953–1954.

(*33*) Belkin, M., Perrault, A., and Shear, M. J.: Tumor necrotizing polysaccharides from plant tissues. (Abstract.) Proc. Am. Assoc. Cancer Res. 2: 4, 1955.

(*34*) Kay, F. W., and Perkin, W. M., Jr.: Experiments on the synthesis of the terpenes. Part IX. The preparation of cyclopentanone-4-carboxylic acid and of cyclohexanone-4-carboxylic acid (δ-ketohexahydrobenzoic acid). J. Chem. Soc. 89: 1640–1648, 1906.

(*35*) Goldin, A., and Mantel, N.: The employment of combinations of drugs in the chemotherapy of neoplasia: *A review*. Cancer Res. 17: 635–654, 1957.

(*36*) Goldin, A., Venditti, J. M., Humphreys, S. R., and Mantel, N.: Comparison of the relative effectiveness of folic acid congeners against advanced leukemia in mice. J. Nat. Cancer Inst. 19: 1133–1135, 1957.

(*37*) Humphreys, S. R., Goldin, A., Venditti, J. M., and Mantel, N.: The influence of amethopterin on the survival time of leukemic mice with respect to food intake, body weight changes, and tumor growth. J. Nat. Cancer Inst. 20: 211–218, 1958.

Studies With Transplantable AK4 Mouse Leukemia. III. Effect of Spleen and Marrow Shielding on AK4 Leukemic Implants in Homologous Strains of Irradiated Mice [1, 2, 3]

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Summary

Alterations in the susceptibility of homologous strains of X-irradiated mice to AK4 leukemic implants by spleen and femur shielding at the time of whole-body irradiation was determined in the A/St and C57BL strains of mice. The protective effect of spleen or femur shielding in irradiated animals that did not receive leukemia was also evaluated in these strains. The results of the experiments with irradiated animals that did not receive leukemia indicated that, although a protective effect was produced by either spleen or femur shielding in both strains, there were no significant quantitative differences afforded by the tissue shielded in either strain. Resistance to transplantable leukemia was partially protected by femur shielding in the irradiated A/St and C57BL mice and by spleen shielding in the irradiated A/St mice. However, in the irradiated C57BL animals that received leukemia, there was no significant difference between the spleen-shielded and nonshielded groups. The differences in the spleen-shielding results between the A/St and C57BL mice tend to indicate that greater protection was associated with myelopoietic tissue than with lymphoid tissue. **J. Nat. Cancer Inst. 21: 513-522, 1958.**

Modification of whole-body X-radiation injury by spleen protection during irradiation (*1*) or by bone-marrow injection post irradiation (*2*) leads to rapid recovery of hematopoietic tissues in mice. These observations have suggested that prolonged depression of hematopoietic tissue may be important in the induction of lymphoid tumors (*3*, *4*). Kaplan and Brown (*5*) were able to prevent the induction of lymphoid neoplasms in C57BL mice by the shielding of one thigh. Lorenz, Congdon, and Uphoff (*6*) effectively inhibited induction of lymphoid tumors in C57BL mice by spleen protection. In contrast, the effect of spleen or marrow shielding on leukemic implants in homologous strains of irradiated mice

[1] Received for publication February 21, 1958.
[2] Work done under the auspices of the U. S. Atomic Energy Commission.
[3] Presented in part at the annual meeting of the Radiation Research Society, June 1956, Chicago, Ill.

has been relatively uninvestigated. This study was undertaken, therefore, to determine whether the susceptibility of irradiated mice to a homologous (AK4) leukemic implant could be altered by spleen or femur shielding at the time of whole-body irradiation. It was also anticipated, through the choice of strains of mice used, that it might be possible to determine the cell types involved if protection should result (*7–9*).

Experimental Methods

Stock leukemia.—AK4 mouse leukemia was employed throughout this study. It was obtained from the Sloan-Kettering Institute [4] and maintained in the AKR strain of mice by weekly transplants. Suspensions of leukemic spleens were used to transplant the leukemia. The preparation of spleen homogenates and method of transplantation were the same as those previously described (*10*). In addition, normal spleen homogenates were prepared as before and were administered to control animals.

Mice.—The inbred A/St and C57BL strains of mice were used. In irradiated mice of these strains it has been possible to demonstrate a linear relationship between the log dose and the probit of percent leukemia "takes" (in this case, equivalent to mortality) 11 days after injection of AK4 leukemia (*10*).

The A/St and C57BL strains of mice were chosen also because of the morphological differences in the hematopoietic activity of the spleen. The spleen of the C57BL mouse has little hematopoietic activity compared to other strains and, as suggested by Dunn (*9*), these morphological differences between the strains may indicate the tissue or cell that is most involved or responsible in the protective mechanisms.

All inbred strains were obtained from the Jackson Memorial Laboratory and consisted of female mice between 8 and 16 weeks of age, with an average age of approximately 12 weeks. The mice were assigned to treatment groups by a system of random numbers prior to radiation exposure.

Exposures.—X-ray exposures were carried out with a 250 kvp Maxitron operated at 250 Kev. and 30 ma., with added Thoraeus II filtration. The radiation under these conditions had a HVL of 2.6 mm. Cu. The target-to-specimen distance was 60 cm., and the dose rate was 53.3 r per minute measured in air. All dose measurements were made with calibrated Victoreen 100 r ionization chambers. Under these conditions the tissue dose to the unshielded portion of the animals was 52.4 rads per minute.

Bone marrow and spleen shielding.—The bone marrow was shielded by a circular lead shield, 20 mm. long and 7 mm. thick, covered with 2 mm. plastic, which was slipped over the right femur of each mouse and held in place with a clamp during exposure. None of the mice in the marrow-shielding experiments was anesthetized. In the spleen-shielding study, all the animals (control and experimental) were anesthetized with a combination of pentothal and chlorpromazine (chlorpromazine 50 mg./kg., sodium pentothal 77 mg./kg.). In the irradiated groups in which the

[4] Memorial Center, New York, N. Y.

spleen was shielded, the spleens were exteriorized (*1*) and placed in grooved, rectangular lead shields that were lined with paraffin. The top of the lead shield covering the spleen was at least 7 mm. thick. Sham-operative surgical procedures were performed on the irradiated control groups in which the spleen was not shielded and on the control group of animals that were unirradiated but inoculated with leukemia or normal spleen homogenate (groups 2, 4, 5, and 6 listed). The plastic and paraffin coverings on the shields were used to absorb the photoelectrons emitted by the lead (*11*).

Radiation doses ranged from 100 to 800 rads and the following groups of 20 mice each were included in each shielding study: 1) irradiated with shielding and inoculated with AK4 leukemia; 2) irradiated without shielding and inoculated with AK4 leukemia; 3) irradiated with shielding and inoculated with normal AKR spleen homogenate; 4) irradiated without shielding and inoculated with normal AKR spleen homogenate; 5) unirradiated and inoculated with AK4 leukemia; 6) unirradiated and inoculated with normal AKR spleen homogenate.

The spleen homogenate inoculation (leukemic or normal) was administered 24 hours after irradiation. The few mice in the spleen-shielding study that died as a result of the surgical procedure were not included in the analysis of the data.

Observations and analysis of data.—The radiation dose required to produce a 50 percent incidence of deaths due to leukemia was calculated on the basis of leukemic deaths at 12 days post irradiation (*10*). The relation between the incidence of leukemic "takes" and radiation dose could be adequately represented by the regression formula of the type:

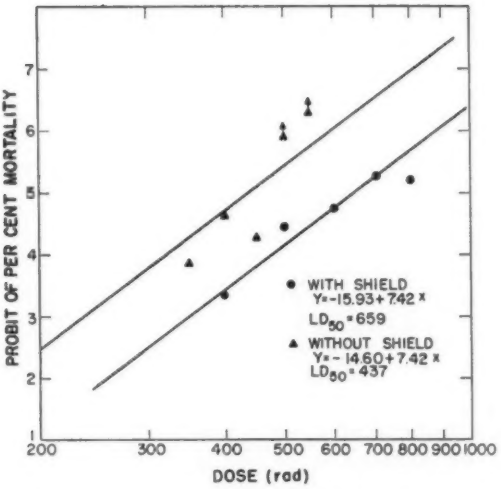
$$Y = a + b \log X$$

where Y = probit of percent leukemic "takes" (or mortality), and X = dose.

The same regression formula was used to calculate LD_{50}^{30} radiation doses in the groups which did not receive leukemia, except that Y = probit of percent mortality due to radiation. Any change in lethality produced by the use of a shield could then be used to determine the efficacy of the shield or as an indirect indication of the physiological mechanisms involved. Variation in lethality was expressed statistically by the apparent change in effectiveness, or relative potency, of the radiation under experimental conditions in which the presence or absence of a shield was the only variable.

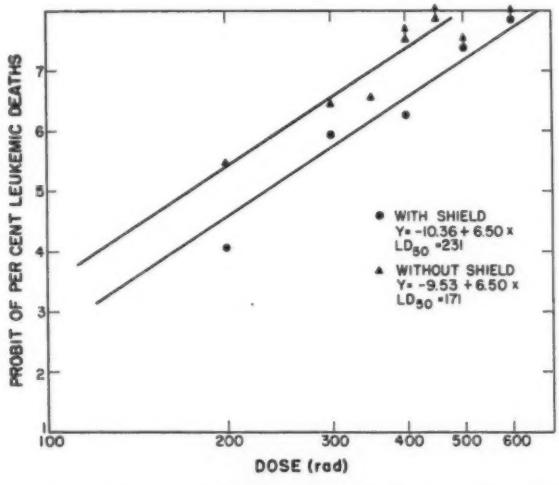
Results

Text-figures 1 to 4 show the regression lines for the results obtained for the A/St and C57BL mice when the probit of percent of deaths due to leukemia or radiation alone was plotted against dose of X rays in the femur-shielding experiments. Text-figures 5 to 8 indicate the regression lines obtained when the same type of analysis was performed on the results

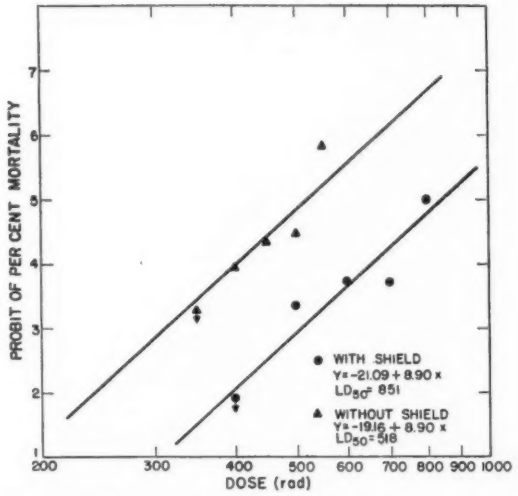


TEXT-FIGURE 1.—Thirty-day mortality response plotted as a function of X radiation of nonshielded and femur-shielded A/St mice.

obtained following spleen shielding of the mice at the time of X-ray exposure. The LD50-30-day mortality due to radiation alone is indicated on text-figures 1, 3, 5, and 7. Spleen and bone-marrow shielding afforded protection in both inbred strains of mice. The A/St mice were equally protected by either femur or spleen shielding. The LD_{50}^{30} values were at least 200 rads higher in the shielded animals compared to the nonshielded animals. Bone-marrow shielding appeared to have a greater protective



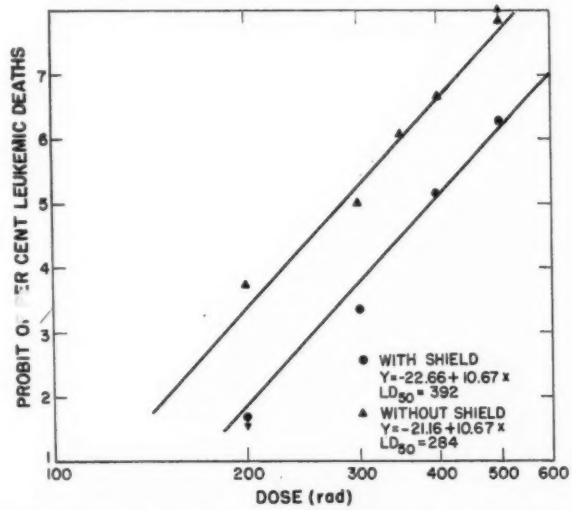
TEXT-FIGURE 2.—Percentage of leukemic deaths 12 days post irradiation (11 days post injection) in nonshielded and femur-shielded A/St mice.



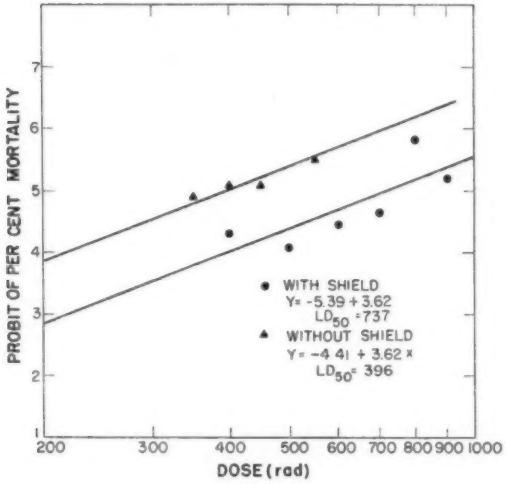
TEXT-FIGURE 3.—Thirty-day mortality response plotted as a function of X radiation of nonshielded and femur-shielded C57BL mice.

effect on the C57BL strains than did spleen shielding. The increase in LD_{50}^{30} dose in the marrow-shielded group was 333 rads over the nonshielded group compared to 226 rads for the spleen-shielded animals.

The LD50-12-day leukemic mortality for both strains is given in text-figures 2, 4, 6, and 8. Abbott's formula and weighting coefficients, described by Finney (*12*), were used in the probit analysis to adjust the

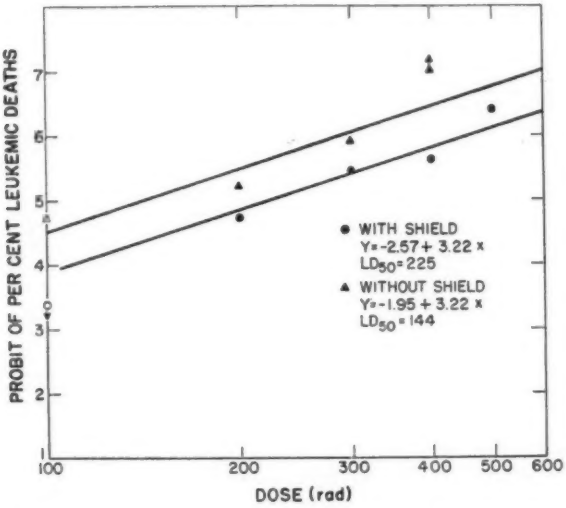


TEXT-FIGURE 4.—Percentage of leukemic deaths 12 days post irradiation (11 days post injection) in nonshielded and femur-shielded C57BL mice.

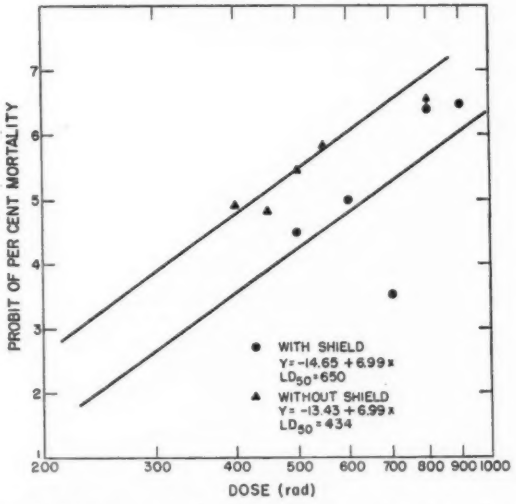


TEXT-FIGURE 5.—Thirty-day mortality response plotted as a function of X radiation of nonshielded and spleen-shielded A/St mice.

data for any mortality in the control groups (*10*). This was necessary in computing the data for text-figures 2, 6, and 8. The LD_{50}^{12} values were always higher in the shielded groups, except in the case of the spleen-shielded C57BL mice that received leukemia (text-fig. 8). In this instance, the difference in 12-day LD50's was not significant and all the points could be represented by one line. In those instances where the



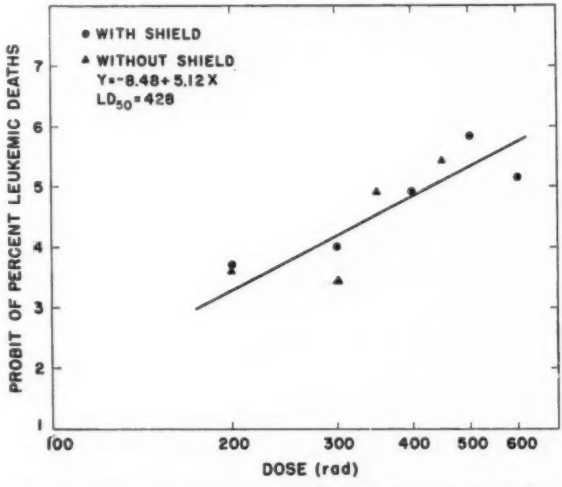
TEXT-FIGURE 6.—Percentage of leukemic deaths 12 days post irradiation (11 days post injection) in nonshielded and spleen-shielded A/St mice.



TEXT-FIGURE 7.—Thirty-day mortality response plotted as a function of X radiation of nonshielded and spleen-shielded C57BL mice.

shielding did afford some protection against leukemia, the increase in LD50 values ranged from 60 to 108 rads. With the exception of the nonspleen-shielded C57BL mice (*see* text-figs. 7 and 8), the LD_{50}^{30} values in the shielded groups that received leukemia were lower than the LD50 values of the irradiated, nonshielded animals that did not receive leukemia.

The relative potency or apparent change in effectiveness of the X radiation is shown in table 1 for the various experimental conditions. In all



TEXT-FIGURE 8.—Percentage of leukemic deaths 12 days post irradiation (11 days post injection) in nonshielded and spleen-shielded C57BL mice.

TABLE 1.—*The relative potency of X radiation in shielded* versus *non-shielded mice*

	Strain of mouse	
	C57BL	A/St
No leukemia		
Spleen shield	0. 67 ± 0. 12	0. 54 ± 0. 08
Femur shield	0. 61 ± 0. 07	0. 66 ± 0. 07
Leukemia		
Spleen shield	0. 95 ± 0. 12	0. 63 ± 0. 13
Femur shield	0. 73 ± 0. 04	0. 78 ± 0. 09

cases, the relative potency is expressed in terms of the ratio of the shielded to the unshielded mice. As the ratio approaches 1, the apparent protection of the shield decreases. The standard errors determined by the usual statistical methods are also shown for each.

The results of the experiments with irradiated animals that did not receive leukemia indicate that, although a protective effect was produced by either spleen or femur shielding in both strains, there were no significant differences afforded by the tissue shielded in either strain. The relative potency values ranged from 0.54 for the spleen-shielded A/St mice to 0.67 for the spleen-shielded C57BL strain. Following femur shielding, the values were 0.66 for the A/St and 0.61 for the C57BL mice.

The results for the experiments with leukemic transplants after irradiation were generally similar, though the relative potency values were slightly higher indicating less protection. However, in the spleen-shielded, leukemic C57BL animals, there was no significant difference in the potency of the X radiation, and this result differed from all other conditions.

Discussion

Spleen and femur shielding have been shown to decrease effectively the acute radiation mortality of C57BL and A/St mice. This response has been shown previously by other investigators (*1*, *2*). In comparing the two strains and two types of shielding used, it was found that the protection afforded did not differ significantly. The results only implied that the spleen shield was more efficacious than the femur shield in the A/St strain, while in the C57BL the reverse appeared to hold. From the results on the irradiated animals it therefore was apparent that the mechanism of protection afforded by either type of shield was qualitatively the same. However, no quantitative significance can be attributed to the results.

In animals injected with transplantable AK4 leukemia following irradiation, the shields again decreased the acute mortality except in the spleen-shielded C57BL mice. The results in the other experiments indicated again that the protection was qualitatively the same. However, the variant result shown by the C57BL mice now gives an index of the quantitative variance of hematopoietic protection. It only becomes apparent through two factors. One factor is the diminished hematopoietic function of the spleen of the C57BL strain which, as suggested by Kaplan and

Paull (*7*), appears to be genetically controlled. This factor has been further discussed by Cole and Ellis (*13*). The second factor is an added stress which, in this instance, is the transplanted leukemia. From these results it appears that quantitative differences in response may be demonstrated by selecting conditions that will emphasize small differences in functional mechanisms.

The spleen-shielding results of this study differ from those reported by Simmons (*14*) in which he found complete protection against DBA leukemia in irradiated, spleen-shielded CF_1 mice with exposures up to 600 r. This would indicate that the quantitative protective effect of the spleen may vary with the strain of mouse and the leukemia used. This is further illustrated by the fact that nonirradiated CF_1 mice treated by Simmons are completely resistant to P1534 leukemia of the DBA/2 mouse, whereas untreated A/St and C57BL mice are not fully resistant to AK4 leukemia. AK4 leukemia may kill from 25 to 35 percent of unirradiated C57BL mice and up to 10 percent of unirradiated A/St mice in 30 days (*10*). As resistance to AK4 leukemia is lower in these two strains, radiation further decreases immune defense mechanisms even in the presence of shielding, so that their ability to resist invading cells is not so great as in the case of P1534 transplants in the CF_1 mouse. The response of a wild strain such as the CF_1 mouse to AK4 leukemia has been so varied (*10*) that it has not been possible to study the effects of shielding.

Histological appearance of the spleen is known to vary with the strain of mouse. Dunn (*8*) has reported that the spleens of strain C57BL mice showed less extramedullary hematopoiesis and a smaller volume of red pulp than spleens of strains C3H and A/St. Kaplan (*15*) examined the shielded spleens of A/St and C57BL mice following whole-body irradiation and noted that hematopoietic elements were more prominent in the shielded spleens of A/St mice. These strain variations in splenic structure may account for the spleen-shielding results obtained in the irradiated A/St and C57BL mice. Greater protection against leukemia was associated with myelopoietic tissue than with lymphoid tissue, indicating that the cells responsible for resistance to transplantable leukemia are the same as those involved in the protection from radiation alone. As indicated by Cole and Ellis (*13*), the bone marrow provides a larger source of these cells than the spleen. But, since the resistance to transplantable leukemia was only partially protected by shielding, it may be that other tissues or cells are involved in the resistance which is not influenced by either spleen or bone-marrow shielding. It is more probable that the volume of tissue shielded was insufficient to respond adequately and to protect the animal completely from such a highly virulent leukemic implant.

Observations of the incidence of *spontaneous* leukemia following irradiation have led to the postulate that prolonged bone-marrow depression accompanied by lymphocytic reduction could be a major factor in induction of lymphoid tumors in mice (*4*, *6*). It would be unlikely that the mechanisms responsible for the decreased incidence of spontaneous

leukemia in the spleen- or femur-shielded irradiated animals (*4*, *5*) would be the same as those responsible for protection seen in the shielded animals given transplantable leukemia.

References

(*1*) Jacobson, L. O., Marks, E. K., Robson, M. J., Gaston, E. O., and Zirkle, R. E.: The effect of spleen protection on mortality following X-irradiation. J. Lab. & Clin. Med. 34: 1538–1543, 1949.

(*2*) Lorenz, E., Uphoff, D., Reid, T. R., and Shelton, E.: Modification of irradiation injury in mice and guinea pigs by bone marrow injections. J. Nat. Cancer Inst. 12: 197–201, 1951.

(*3*) Lorenz, E., Congdon, C. C., and Uphoff, D.: Modification of acute irradiation injury in mice and guinea-pigs by bone marrow injections. Radiology 58: 863–877, 1952.

(*4*) Lorenz, E., Law, L. W., and Congdon, C. C.: The role of bone marrow and spleen in induced and spontaneous lymphatic leukemia. *In* Ciba Foundation Symposium on Leukaemia Research (Wolstenholme, G. E. W., and Cameron, M. P., eds.). 1954, pp. 189–195.

(*5*) Kaplan, H. S., and Brown, M. B.: Further observations on inhibition of lymphoid tumor development by shielding and partial-body irradiation of mice. J. Nat. Cancer Inst. 12: 427–436, 1951.

(*6*) Lorenz, E., Congdon, C. C., and Uphoff, D.: Prevention of irradiation-induced lymphoid tumors in C57BL mice by spleen protection. J. Nat. Cancer Inst. 14: 291–301, 1953.

(*7*) Kaplan, H. S., and Paull, J.: Genetic modification of response to spleen shielding in irradiated mice. Proc. Soc. Exper. Biol. & Med. 79: 670–672, 1952.

(*8*) Dunn, T. B.: Normal and pathologic anatomy of the reticular tissue in laboratory mice, with a classification and discussion of neoplasms. J. Nat. Cancer Inst. 14: 1281–1433, 1954.

(*9*) ———: The importance of differences in morphology in inbred strains. J. Nat. Cancer Inst. 15: 573–589, 1954.

(*10*) Boone, I. U.: Studies with transplantable AK4 mouse leukemia. I. Susceptibility of six heterologous strains of mice after X-irradiation. Radiation Res. 5: 450–458, 1956.

(*11*) Raventos, A.: An abscopal effect of X-ray upon mouse spleen weight. Radiation Res. 1: 381–387, 1954.

(*12*) Finney, D. J.: *In* Probit Analysis: A Statistical Treatment of Sigmoid Response Curve. New York, Cambridge Univ. Press, 1952, pp. 88–99.

(*13*) Cole, L. J., and Ellis, M. E.: On the nature of the spleen-bone marrow radiation recovery factor. USNRDL–TR–23, 1954.

(*14*) Simmons, E. L.: Ability of spleen to protect against leukemia in mice. (Abstract.) Federation Proc. 15: 532, 1956.

(*15*) Kaplan, H. S.: Protection against radiation-induced lymphoma development by shielding and partial-body irradiation of mice. Cancer Res. 12: 441–444, 1952.

Histochemical Demonstration of Acid Phosphatases With Naphthol AS-Phosphates [1]

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Summary

A simple, reliable, and convenient azo-dye method for the demonstration of acid phosphatase and phosphamidase in paraffin-embedded tissues is described. The best results are obtained with frozen-dried tissues. Phosphates of naphthols AS-BI, AS-BS, AS-LC, and AS-TR are useful substrates (text-fig. 1, II). The stability and potential high chromogenicity of these substrates suggest their use in quantitative determinations. The present study indicates that acid phosphatase is not as sensitive an enzyme as previously assumed, and that with reasonable care it can be preserved even in mounted sections over a long period. **J. Nat. Cancer Inst. 21: 523–539, 1958.**

Histochemical procedures for the demonstration of acid phosphatase and phosphamidase are considered to be among the least reliable of enzyme methods (*1*, *2*). This paper describes an azo-dye procedure for the demonstration of acid phosphatase and possibly phosphamidase. The technique results in completely consistent and reproducible histochemical localizations in paraffin sections. In addition, synthesis of the substrates is convenient and inexpensive. The success of the procedure depends on the use of new substrates that are highly stable derivatives of hydroxynaphthoic acid anilides. These compounds, on enzymatic hydrolysis, release exceedingly insoluble naphthols, which couple efficiently at an acid *p*H and thus enable good microscopic localizations.

Methods

Synthesis of Substrates

Phosphates of naphthol AS-TR(I), AS-BI(II), AS-BS(III), AS-MX, AS-P, and AS-LC (text-fig. 1) were prepared as the previously described

[1] Received for publication February 24, 1958.

[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

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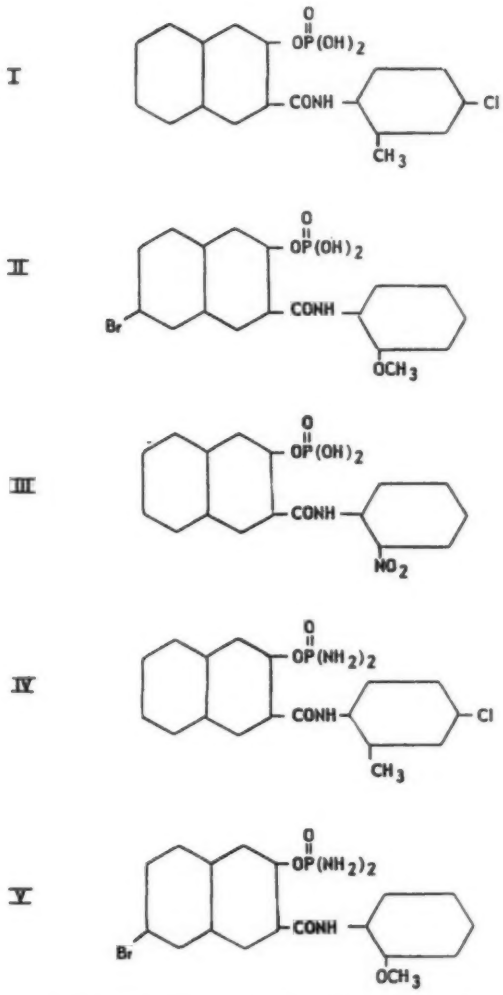


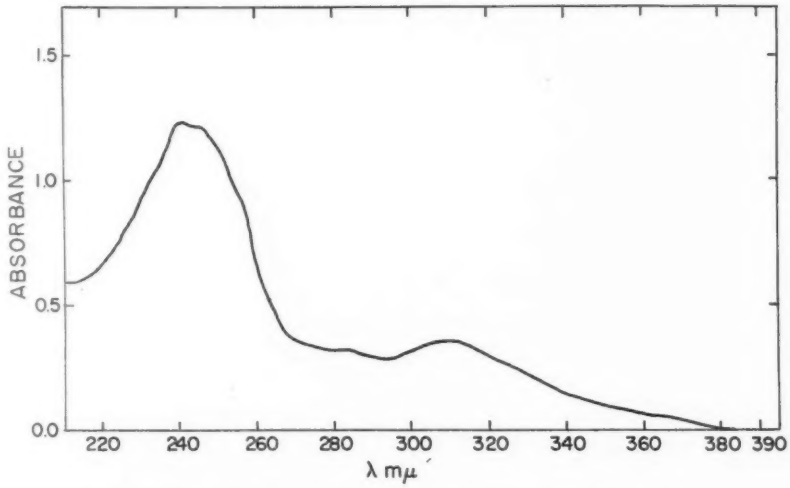
TEXT-FIGURE 1.—Structural formulas of AS-phosphates and phosphodiamides. Structural formulas of AS-naphthols may be found in reference *4*.

procedure (*3*). The naphthols [3] were first recrystallized from hot DMF (*N,N*-dimethylformamide)-ethanol. Four to 5 gm. (0.011 to 0.02 M) of naphthol were suspended in 50.0 ml. tetrahydrofuran (Matheson, Coleman, and Bell) (some commercial samples require redistillation) with 300 to 400 mg. Versene (EDTA, tetrasodium salt), 50 mg. sodium lauryl sulfate, 3.0 gm. sodium chloride, and an excess of phosphorus oxychloride (approximately 4.0 ml.). Two ml. of pyridine were then added rapidly

[3] Naphthols furnished through courtesy of Pfister Chemical Works, Ridgefield, N. J., and Verona Dyestuffs, Springfield, N. J.

(within a period of 2 to 3 seconds) with constant stirring. The mixture was immediately evaporated to a syrup or slush under a jet of air and a cube or two of ice added with constant stirring. The reaction mass was then added to 500.0 ml. cold water, stirred, and collected under suction. For histochemical demonstration the acid phosphates were used without conversion to their sodium salts. As previously indicated, these compounds may be prepared at a cost of less than one dollar an ounce (*3*). Naphthol AS-TR and AS-BI phosphodiamides (text-fig. 1 : IV, V) were prepared by treating the appropriate naphthyl phosphochloridate in tetrahydrofuran with a slight excess of aqueous ammonia. The gelatinous precipitate that formed was discarded; the supernatant evaporated to dryness and revealed a formation of fine crystals. Other basic amines (methylamine, butylamine, triethylamine) were also used to prepare more complex phosphodiamides. These compounds were not thoroughly tested. They were found, however, to be hydrolyzed by kidney sections and gave good localizations.

Ultraviolet and infrared absorption spectrums[4] of several of the acid phosphates were observed. The spectrums of both the naphthol and its corresponding phosphate were similar (text-figs. 2 and 3). In the case of naphthol AS-BI phosphate the ultraviolet spectrum showed maxima at 241, 282, and 308 mμ.

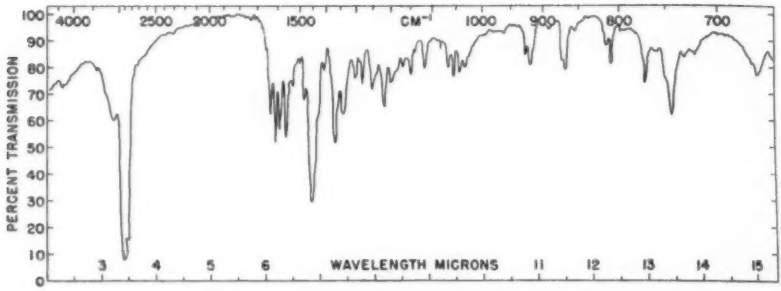


TEXT-FIGURE 2.—Ultraviolet spectrum of AS-BI phosphate. (Substrate concentration .0140 mg. per ml. ethanol.)

Histochemical Techniques

For histochemical use, substrates, approximately 4.0 mg., were dissolved in 0.25 ml. *N*, *N*-dimethylformamide (DMF) (Matheson, Coleman, and

[4] Courtesy of Dr. W. Wildman, Instrument Laboratory, Laboratory of Chemistry of Natural Products, National Heart Institute.



TEXT-FIGURE 3.—Infrared spectrum of AS-BI phosphate (Nujol mull).

Bell)—the least amount of solvent practicable is best—followed by 25.0 ml. distilled water and 25.0 ml. of buffer (0.2 M acetate, *p*H 5.2–5.6) to make a total volume of 50.0 ml. In frozen sections, 0.1 ml. of solvent was used. Thirty-five mg. of diazonium salt (Red Violet LB salt,[5] diazotized 5-benzamido-4-chloro-2-toluidine) and 2 drops of 10 percent $MnCl_2$ were added. The mixture was then shaken and filtered into a coplin jar. Incubation times varied from 30 minutes to many hours depending upon the activity of the tissue and the type of fixation employed. Substrates derived from naphthols AS-BI, AS-TR, and AS-MX are particularly stable, and solutions remained crystal clear even after many hours at room temperature. Incubation at 37° C. may be employed for periods of several hours without affecting the stability of the substrate solution or producing a background stain. In less active tissues (acetone-fixed paraffin-embedded) slides may be incubated overnight without any visible deterioration of the substrate solution.

The tissues used consisted of cold acetone-fixed paraffin-embedded tissues, and both single and double embedded frozen-dried material of several species. Rat tissues consisted of kidney, liver, pancreas, spleen, lymph node, stomach, intestine, and epididymis. Tissues from mouse kidney, liver, and pancreas were also studied, as well as tissues from several transplanted mouse osteogenic sarcomas. The human tissues consisted of thyroid, adrenal, prostate, and salivary glands (submaxillary), spleen, intestine, and inflammatory tissue (granuloma). Sections of several human neoplasms were also observed. These included carcinoma of the lung, epidermoid carcinoma, carcinoma of the stomach, ganglioneuroma, and Hodgkin's disease (lymph node).

Paraffin-embedded tissues were dewaxed with petroleum ether or xylene, which was followed by several changes of acetone prior to incubation.

An attempt was made to characterize the histochemical reaction with potential enzyme inhibitors which were interposed during the hydrolysis period. These included 0.001 M concentrations of the following: $(NH_4)_6Mo_7O_{24}$ (*5*), KCN, sodium arsanilate (*6*), tartaric acid (*7*), $ZnSO_4$,

[5] Furnished through courtesy of Verona Dyestuffs, Springfield, N. J.

$CuSO_4$, and NaF (0.01 M). For these tests no activator (Mn) was used. Representative sections were also placed in hot water (90° C.) for 5 minutes, which resulted in complete inactivation of the enzyme.

Observations

Excellent results were obtained with all types of fixed tissues. Frozen-dried tissues gave uniform and very sharp localizations. The staining reaction was consistently uniform, and patchy or irregular staining was never observed. Tissues never failed to stain, which does occur with the glycerophosphate method. Among the many hundreds of sections stained, nuclear staining was never observed. The best localizations were obtained with frozen-dried paraffin-embedded tissues. Good results were obtained even with acetone-fixed paraffin-embedded sections that had been sectioned and mounted 3 or 4 years previously. The following microscopic observations pertain primarily to the use of the BI- and TR-phosphates and the TR-phosphoamide on frozen-dried double and single embedded tissues, unless otherwise indicated. The other substrates gave similar localizations with minor variations. The possibility that such variations indicate different substrate specificities warrants additional study. The TR-phosphoamide was used only in conjunction with rat kidney and liver.

Rat and Mouse Tissues

The kidney revealed a strong cytoplasmic reaction of both proximal and distal convoluted tubules as well as brush-border staining. Numerous cytoplasmic granules were visible (fig. 8). Sections of acetone-fixed rat and dog kidney, which had been prepared several years previously, exhibited considerable activity and stained uniformly. With the AS-TR phosphoamide, a finely granular reaction was obtained which differed from the acid-phosphate reaction in that a primarily basal localization with no brush-border staining was observed.

The reaction in liver with all substrates was granular in appearance and was localized primarily to bile canaliculi (fig. 9). (This distribution may be of diagnostic significance in liver disease.) Mouse liver revealed only staining of leukocytes.

The pancreas revealed some acinar staining after long incubation periods (overnight).

Both spleen and lymph node contained large numbers of reactive macrophages and reticular cells. In the spleen the red pulp showed the greatest staining reaction.

The stomach did not stain. However, the adjacent duodenum revealed an intense reaction at the borders of the villi.

The columnar epithelium of the epididymis stained intensely (fig. 1). A comparison with the alkaline-phosphatase reaction is seen in figure 2.

Highly reactive giant cells were present in the mouse osteogenic sarcoma (fig. 10). In addition, foci of calcification revealed phosphatase activity of the matrix.

Human Tissues

The thyroid showed staining of both colloid and epithelium. The latter contained discrete cytoplasmic granules (fig. 3).

Human intestine (ileum) stained like that of the previously described rat duodenum.

Prostatic epithelium stained intensely (fig. 4). The epithelium also revealed intensely staining granules. The prostatic secretion that was preserved in the frozen-dried material also stained intensely (fig. 5).

Some of the salivary-gland (submaxillary) ducts contained reactive cytoplasmic granules.

The spleen revealed essentially the same reaction as that of its rat counterpart.

All zones of the adrenal parenchyma stained. The most prominent reaction was confined to the reticular zone. The dark cells contained positive-staining granules and droplets.

Human Inflammatory and Neoplastic Tissues

The dental granuloma revealed a prominent staining reaction of macrophages and giant cells. Of interest is the fact that the inflammatory lesion at this stage is considered to be at an acid *p*H (*8*).

The lung carcinoma showed groups and masses of intensely staining neoplastic cells. If this is a common characteristic of lung tumors, it could prove to be a useful diagnostic tool in the study of bronchial aspirations.

The epidermoid carcinoma (base of tongue) revealed intense staining of dyskeratotic cells comprising "pearls" (fig. 11). Other areas of the neoplasm did not stain.

The gastric carcinoma showed a granular pattern of activity confined primarily to the basal portion of tumor cells (fig. 6).

The ganglioneuroma contained groups of intensely staining "ganglion cells" (fig. 7).

All components of the Hodgkin's lymph node stained. The reaction was particularly intense in Sternberg-Reed cells.

Inhibitor Studies

The following compounds were found to inhibit the reaction: $(NH_4)_6Mo_7O_{24}$, sodium arsanilate, tartaric acid, and sodium fluoride.

Discussion

Even though hundreds of papers have been written about the acid-phosphatase reaction since Gomori first described the procedure in 1941, the technique still remains as one of the controversial areas of histochemistry. Aspects of this problem have been well reviewed by Gomori (*1*), Pearse (*2*), and Novikoff (*9*).

Factors such as nonspecific staining of nuclei and axons have been attributed to lead impregnation of these structures. In addition to the

problem of nonenzymatic staining, Gomori lists so-called "undetermined factors" (*1*). "This group includes the queerest types of failures, unexplainable by any of the causes enumerated. To mention only a few examples: sections cut from a single block stain uniformly one day but utterly refuse to stain the next day, although the incubating mixture is prepared in exactly the same way both times; out of a ribbon of several serial sections, mounted on one slide, one or two stain exceedingly poorly while the majority show an excellent reaction." Goetch and Reynolds claim that two factors radically affect the method (*10*). These include the technique of mounting the section as well as that of dewaxing and hydration. They state that too high a water-bath temperature causes inactivation. In addition, they claim that the trace of egg albumin used in mounting sections has an inhibitory effect upon the enzyme. They recommend that sections not be dewaxed since this process ostensibly affected the enzyme.

Eränko stated that acetone-fixed paraffin-embedded tissues were unsatisfactory for use with the Gomori technique and recommends that the technique only be used with fresh-frozen or formalin-fixed frozen tissue (*11*). Pearse also recommends the use of formalin-fixed frozen sections and claims that the results with frozen-dried sections are not as good as those with frozen sections (*2*). Pearse also considers acetone fixation and paraffin embedding as unsatisfactory. He continues by saying, "Since the distribution of precipitate has thus been shown to be variable, depending on physicochemical conditions rather than on enzyme distribution, it is necessary to conclude that the lead phosphate technique, at best, gives only an approximate or topographical picture of the distribution of acid phosphatase in the tissues."

Glück states that the enzyme can cease to be histochemically demonstrable if the tissue is allowed to stand for more than 4 hours at a temperature of 20° C. before fixation (*12*). Many of the acetone-fixed tissues described in the present work were autopsy specimens which remained at room temperature or above for a number of hours. Notwithstanding, the histochemical reaction was quite satisfactory. The author does not necessarily recommend autopsy tissues for enzyme studies, but for cursory study, they appear to be entirely satisfactory.

Attempts to use azo-dye techniques to demonstrate acid phosphatase have been satisfactory only on a rather gross level (*2*). This is due to the fact that simple naphthols such as α-naphthol couple poorly at *p*H 5 (*13*). Pearse reported, however, that with the azo-dye technique nuclear staining was not visible (*2*). Burton also reported on the use of α-naphthyl phosphate for demonstration of acid phosphatase (*14*). According to Rutenberg and Seligman, sections stained with this technique revealed lack of histological detail and diffusion artifacts exemplified by nuclear staining and furry outlines of stained cells (*15*).

Despite the vagaries of the original glycerophosphate technique, one does gain the impression that acid phosphatase is a labile enzyme readily subject to inactivation from any number of procedures encountered

in the histological processing of tissues. The present work indicates that this view is not correct and that acid phosphatase readily withstands a number of fixation and embedding procedures. Among the many hundreds of slides stained by the present technique, the author has not experienced a single failure or patchy reaction. Frozen-dried material, either singly or doubly embedded, yields excellent localizations and is most suitable for use with the AS substrates. However, even cold acetone-fixed paraffin-embedded tissues give good results. The validity of the present technique is further suggested by comparison with biochemical data, which indicate, for example, that liver phosphatase is cytoplasmic and not nuclear (*9*, *16*).

Of interest is the comment of Gomori, who states, "It is certain that a significant diffusion of enzyme (acid phosphatase) takes place from formalin-fixed frozen sections, and even more from unfixed cryostat sections" (*13*). De Duve and his group have also shown that acid phosphatase is readily made soluble and is released from tissues by freezing and thawing (*17–19*). Their work would also suggest the desirability of using fixed tissues for the histochemical demonstration of acid phosphatase. They also claim that acid phosphatase (liver) is associated with cytoplasmic granules of unknown cytological nature (*19*, *20*). Possibly the granules shown in figures 8 and 9 may correspond in part to the aforementioned particles.

Gomori states that the original phosphamidase technique employing *p*-chloroanilidophosphoric acid is not dependable. The difficulty stems not only from the presence of lead ions, as with the acid-phosphatase technique, but also from the fact that the substrate is unstable. Slides must be incubated, inverted, and at an angle to avoid accumulation of precipitate. Even then, patchy staining or failures may result. Phosphoamides of another type are known to be hydrolized by phosphamidase (*21*, *22*). One of these has the structure $R\text{-}OPO(NH_2)_2$ (*e.g.*, Naphthol AS-TR phosphodiamide, text-fig. 1 : IV). As with the other AS-phosphates, this substrate is highly stable and gives consistently reproducible localizations. However, more biochemical and histochemical evidence is needed to elucidate the substrate specificities of phosphamidase.

The rationale of the use of arylamide derivatives of hydroxynaphthoic acid for precise localizations has been previously discussed (*3*). These very substantive compounds azo-couple efficiently over a wide *p*H range to form brilliantly colored, highly insoluble azo dyes of noncrystalline structure, at the cytological level. In general the most precise localizations are obtained with complex derivatives such as AS-BI and AS-LC (the 4-chloro-2,5-dimethoxyanilide) phosphates. The latter is the counterpart of AS-LC acetate, an esterase substrate (*23*).

Holt states, "It is reasonable to conclude that better localization is given by the less soluble dyes or those of higher substantivity" (*24*). AS-naphthols are among the most substantive and insoluble naphthols known. A comparison of the solubilities (aqueous) of various naphthols proposed for acid phosphatase substrates may be of interest. These solubilities,

partly based on the data of Gomori (*13*), are given in micrograms per milliliter:

α-naphthol	303
6-Br-2-naphthol	195
6-benzoyl-2-naphthol	9.0–27.0
2,4-dichloro-1-naphthol	12.0
naphthol AS and complex derivatives	<1.0

One factor of considerable importance, which has been overlooked in the past, is the choice of diazonium salt. Investigators have often chosen diazonium salts at random or have used one particular salt. Diazo Blue B, for example, is commonly used, even though it is a slow coupler and recommended only for use in the alkaline range (*25–27*). The Variamine Blue series of salts are also slow couplers and exhibit marked decomposition when a *p*H value of 8.5 is exceeded. Hees points out that the strongly positivizing aminophenyl grouping in a diazonium salt (*e.g.*, diazotized aminodiphenyl amines) results in pronounced coupling sluggishness. In addition, electropositive substitutions (OCH_3) ortho to the diazonium grouping further decreases coupling speed. Naturally, in a double ortho-substitution of a diamine, the positivizing effect of the methoxy group is especially pronounced (*25*).

In order to facilitate good localizations it is desirable to use a diazonium salt with a higher coupling energy. These salts contain electronegative groups in the molecule, *e.g.*, NO_2, Cl, or Br, which augment the coupling rate. The higher the coupling energy, the more the *p*H range can be shifted toward the acid side while still maintaining rapid coupling. For example, diazotized *m*-nitroaniline, 4-chloro-2-nitroaniline, and 2,5-dichloroaniline couple rapidly at *p*H 4 to 5 with AS-naphthols (*25*, *26*). Red Violet LB salt, which was used in the present study, is a good example of a compound containing an electronegative halogen grouping, an amide grouping, and an additional benzene ring. The sum total of these factors adds up to rapid coupling and high chromogenicity of the final dyestuff.

The type of naphthol also exercises a certain influence on the coupling rate. The influence of the naphthol component is, however, less marked and, aside from special exceptions, is overshadowed by that of the diazonium salt.

References

(*1*) Gomori, G.: Microscopic Histochemistry: Principles and Practice. Chicago, Univ. Chicago Press, 1952, 273 pp.

(*2*) Pearse, A. G. Everson: Histochemistry: Theoretical and Applied. Boston, Little, Brown & Co., 1953, 530 pp.

(*3*) Burstone, M. S.: Histochemical comparison of naphthol AS-phosphates for the demonstration of phosphatases. J. Nat. Cancer Inst. 20: 601–615, 1958.

(*4*) The Chemistry of Synthetic Dyes and Pigments (Lubs, H. A., ed.). New York, Reinhold Publishing Corp., 1955, 734 pp.

(*5*) Courtois, J., and Bossard, M.: Action des inhibiteurs sur la phosphatase d'amande. IV. Action des molybdates. Bull. Soc. Chim. Biol. 26: 464–469, 1944.

(6) ROCHE, J.: Phosphatases. *In* The Enzymes (Sumner, J. B., and Myrbäck, K., eds.). New York, Academic Press, Inc., 1950, 728 pp.

(7) KILSHEIMER, G. S., and AXELROD, B.: Inhibition of prostatic acid phosphatase by α-hydroxycarboxylic acids. J. Biol. Chem. 227: 879–890, 1957.

(8) MENKIN, V.: Newer Concepts of Inflammation. Springfield, Illinois, Charles C Thomas, 1950, 145 pp.

(9) NOVIKOFF, A. B.: Histochemical and cytochemical staining methods. *In* Analytical Cytology (Mellors, R. C., ed.). New York, McGraw-Hill Book Co., Inc., 1955, chapt. 2, p. 1–63.

(10) GOETSCH, J. B., and REYNOLDS, P. M.: Obtaining uniform results in the histochemical technic for acid phosphatase. Stain Technol. 26: 145–151, 1951.

(11) ERÄNKO, O.: Histochemical evidence of the presence of acid-phosphatase-positive and -negative cell islets in the adrenal medulla of the rat. Nature, London, 168: 250–251, 1951.

(12) GLÜCK, E.: Les phosphomonoesthérases des néoformations mammaires. Etude histochimique. Acta pathol. et microbiol. scandinav. (supp.): 93: 106–120, 1952.

(13) GOMORI, G.: Histochemical methods for acid phosphatase. J. Histochem. 4: 453–461, 1956.

(14) BURTON, J. F.: Histochemical demonstration of acid phosphatase by an improved azo-dye method. J. Histochem. 2: 88–94,1954.

(15) RUTENBURG, A. M., and SELIGMAN, A. M.: The histochemical demonstration of acid phosphatase by a post-incubation coupling technique. J. Histochem. 3: 455–470, 1955.

(16) PALADE, G. E.: Intracellular localization of acid phosphatase; a comparative study of biochemical and histochemical methods. J. Exper. Med. 94: 535–548, 1951.

(17) BERTHET, J., BERTHET, L., APPELMANS, F., and de DUVE, C.: Tissue fractionation studies. 2. The nature of the linkage between acid phosphatase and mitochondria in rat-liver tissue. Biochem. J. 50: 182–189, 1951.

(18) APPELMANS, F., and de DUVE, C.: Tissue fractionation studies. 3. Further observations on the binding of acid phosphatase by rat-liver particles. Biochem. J. 59: 426–433, 1955.

(19) GIANETTO, R., and de DUVE, C.: Tissue fractionation studies. 4. Comparative study of the binding of acid phosphatase, β-glucuronidase and cathepsin by rat-liver particles. Biochem. J. 59: 433–438, 1955.

(20) APPELMANS, F., WATTIAUX, R., and de DUVE, C.: Tissue fractionation studies. 5. The association of acid phosphatase with a special class of cytoplasmic granules in rat liver. Biochem. J. 59: 438–445, 1955.

(21) ICHIHARA, M.: Über die phosphamidase. J. Biochem. 18: 87–106, 1934.

(22) MØLLER, M.: On the phosphamidase activity of human seminal phosphate. Biochem. et biophys. acta. 16: 162–165, 1955.

(23) BURSTONE, M. S.: The cytochemical localization of esterase. J. Nat. Cancer Inst. 18: 167–172, 1957.

(24) HOLT, S. J.: The value of fundamental studies of staining reactions in enzyme histochemistry, with reference to indoxyl methods for esterases. J. Histochem. 4: 541–554, 1956.

(25) HEES, W.: Die Azofarbstoffkupplung in der Naphtol AS-Färberei I. Melliand Textilberichte 30: 359–363, 1949.

(26) ———: Die Azofarbstoffkupplung in der Naphtol AS-Färberei II. Praktische Kuppelungs-bedingungen in der Naphtol AS-Färberei. Melliand Textilberichte 30: 525–528, 1949.

(27) ———: Die Azofarbstoffkupplung in der Naphtol AS-Färberei III. Therortische Berechung der Ph-Werte von Entwicklungsslotten. Melliand Textilberichte 31: 47–48, 1950.

PLATES

PLATE 62

All specimens frozen and dried. Substrate is naphthol AS-BI phosphate unless otherwise indicated.

FIGURE 1.—Rat epididymis showing activity of epithelium—3-hour incubation. × 143

FIGURE 2.—Rat epididymis (alkaline phosphatase) showing difference in distribution as compared with figure 1—45-minute incubation. AS-MX phosphate. × 143

FIGURE 3.—Human thyroid showing granular distribution of activity—6-hour incubation. × 285

FIGURE 4.—Human prostate. Cytoplasm stains intensely. In addition, there are discrete (droplets) zones of staining. One-hour incubation. × 143

Photomicrographs taken by Mr. John McGuire

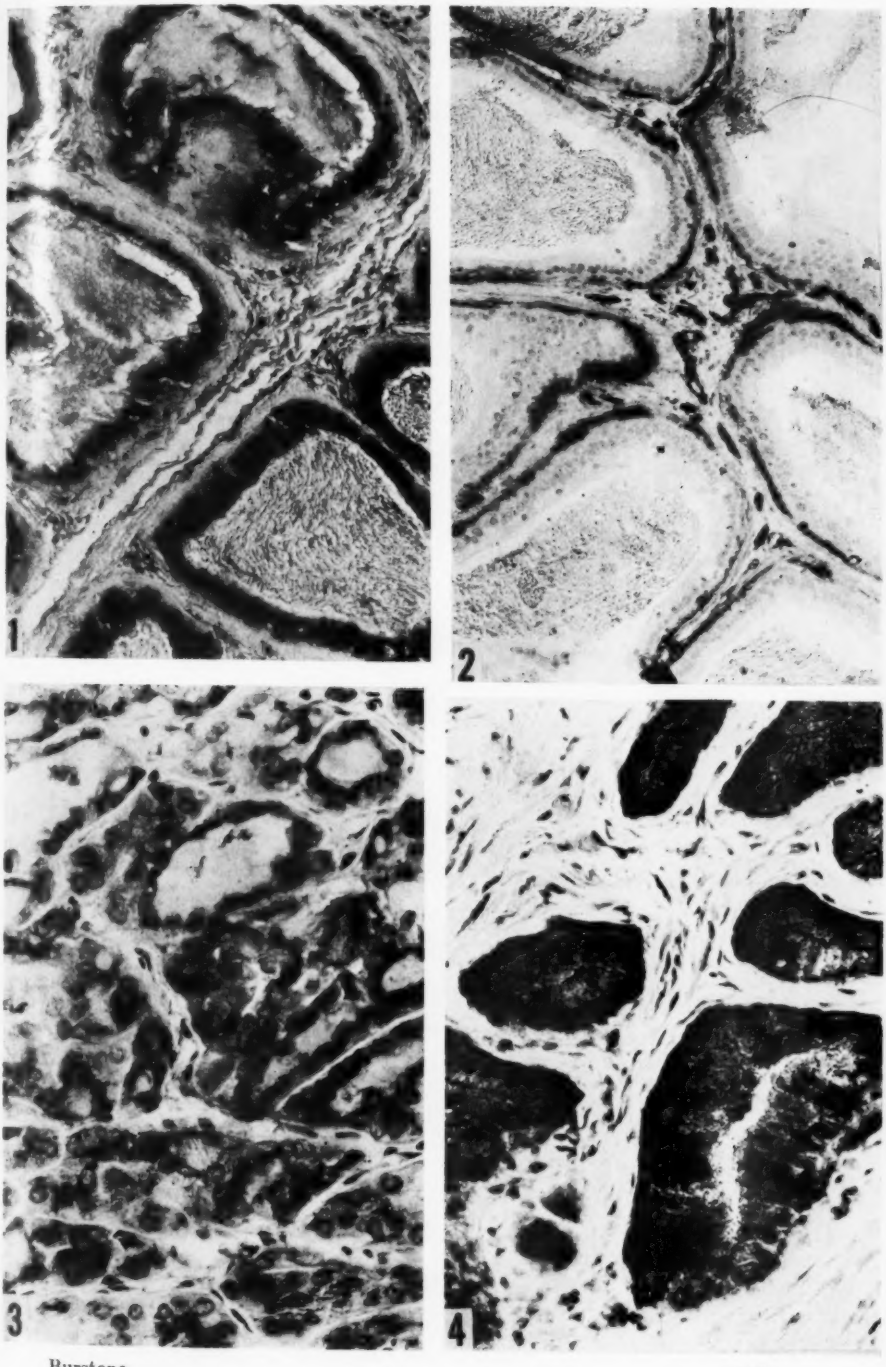

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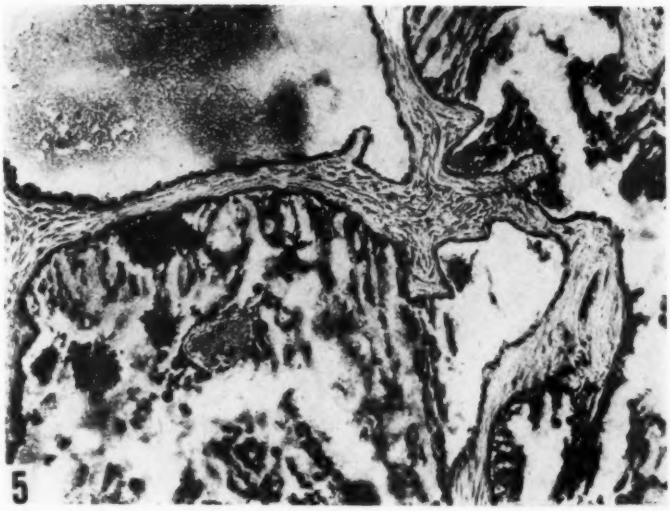
PLATE 63

FIGURE 5.—Human prostate showing reactivity of prostatic secretion—1-hour incubation. × 143

FIGURE 6.—Gastric carcinoma. There is a granular distribution of activity primarily localized to the basal portions of tumor cells—2-hour incubation. × 143

FIGURE 7.—Ganglioneuroma from suprarenal area—2-hour incubation. AS-BS phosphate. × 143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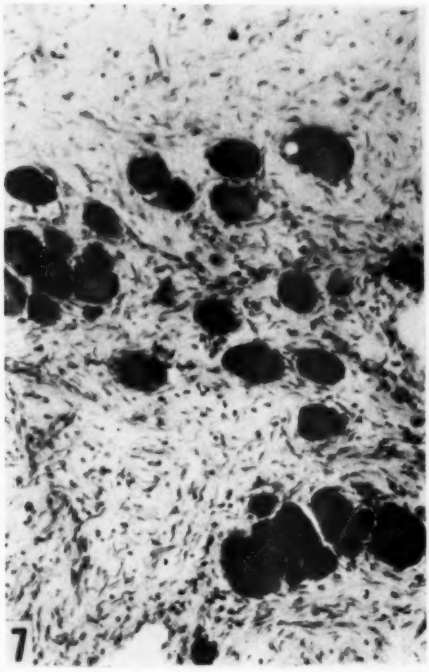
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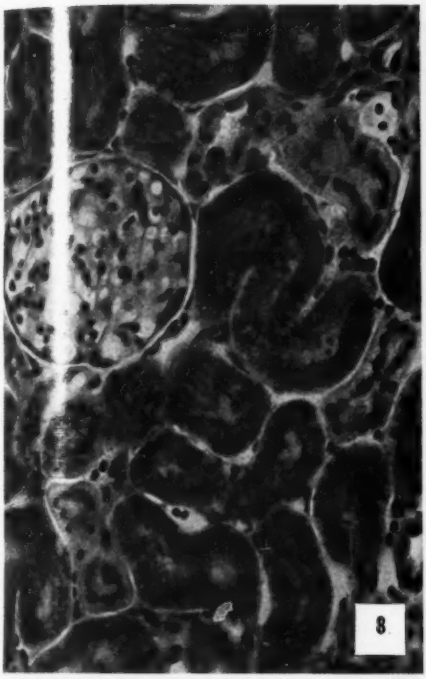
PLATE 64

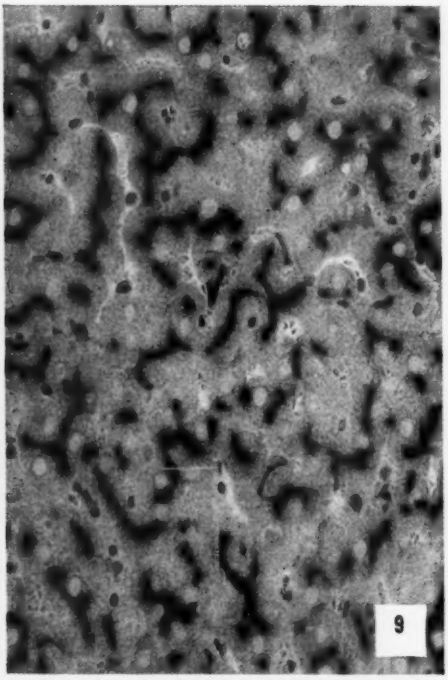
FIGURE 8.—Rat kidney. There is strong cytoplasmic activity of tubular epithelium. *Note* granular distribution of activity. Six-hour incubation. × 285

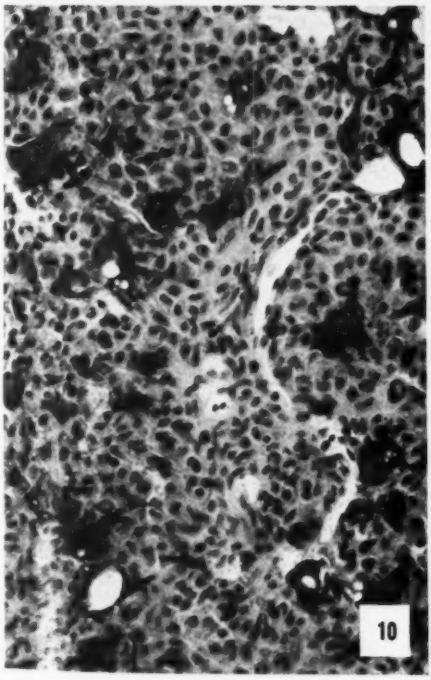
FIGURE 9.—Rat liver. Activity outlines bile canaliculi—4-hour incubation. × 285

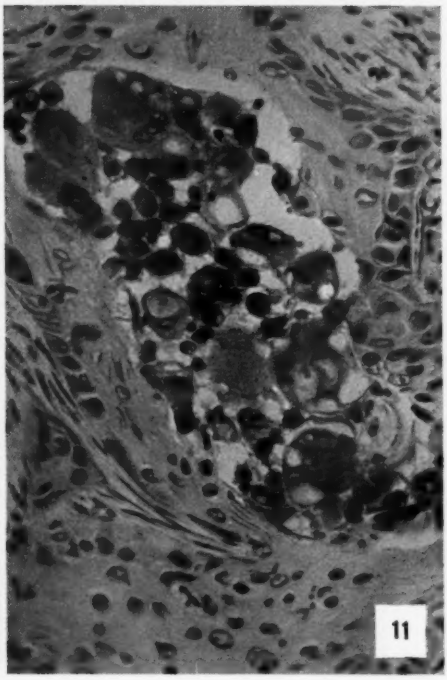
FIGURE 10.—Mouse osteogenic sarcoma showing intense reaction of giant cells—2-hour incubation. (Specimen furnished by Dr. Thelma Dunn.) × 210

FIGURE 11.—Epidermoid carcinoma (base of tongue). Dyskeratotic cells show activity—3-hour incubation. × 285









Burstone

The Effects of Heterologous Immune Serums upon HeLa Cells *in Vitro* and Rat-HeLa Tumors *in Vivo* [1,2]

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Summary

Heterologous immune serums against HeLa cells were prepared in rabbits, rats, and a tuberculin-sensitive cow. The effects of these immune serums *in vitro* upon HeLa cells were studied by means of a cytotoxic metabolic inhibition test (CMI), the complement-fixation test, and by agglutination tests. The CMI test was sensitive, quantitative, and reproducible. The cytotoxic activity of the cow immune serum was heat resistant, removed by heating to 60° C. for 30 minutes, restored by adding fresh unheated serum, and demonstrated a number of properties compatible with the presence of a specific cytotoxic antibody. The protective effect *in vivo* of the cow immune serum was studied by the quantitative and reproducible rat-HeLa tumor technique. Good correlation was obtained between the number of HeLa cells killed in the CMI test and the number killed *in vivo* in the rat-protection tests. **J. Nat. Cancer Inst. 21: 541–555, 1958.**

The effects of heterologous immune serums upon cancer have been studied in the past by a number of *in vitro* and *in vivo* techniques. As reviewed by Hauschka (*1*), Mohos and Kidd (*2*), Wissler and Flax (*3*), and Ross (*4*), the work of early investigators provided little evidence that heterologous immune serums were active or specific against the cancer cells used as antigen. More recently, however, some evidence has appeared indicating the effectiveness and specificity *in vivo* and *in vitro* of some heterologous immune serums against the cancer cell studied (*2, 3, 5–14*). With the aid of modern tissue-culture techniques some human cancer cells can be maintained in pure culture in continuous passage in a defined medium, which makes possible a degree of quantita-

[1] Received for publication March 12, 1958.
[2] Aided by a grant from the National Foundation for Infantile Paralysis.
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tion and reproducibility of laboratory tests not available to earlier workers. In the experiments described here, such techniques have been applied, and heterologous antiserums were shown to be effective on cancer cells both *in vitro* and *in vivo*. The human tissue culture cell line, HeLa (*15*, *16*), was used as a cancer-cell antigen for the production of immune serums in rabbits, rats, and a cow. The technique for measuring the *in vitro* cytotoxic activity of *homologous* immune serums, described by Salk (*17*), was used in our studies to note the cytotoxic effects of *heterologous* immune serums upon HeLa cells. The *in vivo* cytotoxic activity of these same heterologous serums was measured by a technique, to be described, which used the X-irradiated cortisone-treated rat described by Toolan (*18*, *19*) and modified by Suskind and his associates (*20*) and Coriell and his associates (*21*) and in which prompt and regular production of large solid intraperitoneal tumors was observed following the intraperitoneal injection of HeLa cells.

Materials and Methods

Cell cultures.—HeLa cells were maintained in continuous passage in milk dilution, Blake and Povitsky bottle cultures. The HeLa cell cultures were received from Microbiological Associates approximately 1 year before these experiments. The nutrient medium consisted of 80 percent medium 199 (*22*), 20 percent human or calf serum, and penicillin, streptomycin, and nystatin (Mycostatin, E. R. Squibb & Sons) in a final concentration of 100 units, 100 μg., and 25 units per ml., respectively. The tissue-culture techniques used in this laboratory have been described (*23*).

HeLa cell suspensions for tumor production in rats were prepared by removing the cell sheets from 6- to 7-day Povitsky bottle cultures with 50 ml. of 0.25 percent Versene [6] (*24*), centrifuging at 1000 r.p.m. for 10 minutes, resuspending the cells in medium 199, and enumerating the viable cells using 1:400 neutral red. The cell suspensions were adjusted to contain 0.5 to 5 $\times$ 10^6 cells per ml. Rats were injected intraperitoneally with 2 ml. of these suspensions containing 1 to 10 million HeLa cells.

Female albino rats (Wistar or Osborne-Mendel, Barkbridge Farms) of weanling age and 45 to 55 gm. in weight received one dose of 250 r total-body X radiation 1 to 4 days prior to cell inoculation, and 5.0 mg. of cortisone acetate (Cortone, Merck Sharp & Dohme) injected intramuscularly immediately following cell inoculation and every 2 to 3 days for a total of 5 doses. The physical factors of radiation were as follows: 240 kv., ½ mm., Cu+1 mm. Al filter, HVL 1.2 cm. Cu, field size 20 $\times$ 20 cm., TSD 50 cm, average dose 250 r. The rats were observed daily for 21 days following cell inoculation when all survivors were killed and examined for gross and microscopic evidence of tumor formation. All rats received antibiotics prophylactically to suppress bacterial infections. Chloramphenicol [7] (0.16 mg./ml.) or Paromomycin [8] (0.5 mg./ml.) were added to

[6] EDTA, Hartman-Leddon Co., Philadelphia, Penna.
[7] Parke, Davis & Company.
[8] Humycin, Parke, Davis & Company.

the milk in all experiments and some rats also received 5,000 units of penicillin injected intramuscularly every 2 days.

In these experiments, groups of 12 to 20 rats were used for each variable of the test and an equal number observed as controls. The over-all death rate during the first 3 weeks after cell inoculation was 35 percent. Only rats surviving 10 or more days after cell inoculation were included in the results. HeLa-rat tumors in this laboratory reach a maximal size approximately 21 days after HeLa cell inoculation when the rats are usually killed. In those permitted to survive, the tumors slowly regress over a period of 6 months. Prior to these experiments, 780 out of 811 (96%) of the X-irradiated and cortisone-treated rats had developed abundant tumors 10 to 21 days after injection intraperitoneally with 5 to 10 $\times$ 10^6 HeLa cells.

Pathology.—The gross appearance of the intraperitoneal tumors at autopsy was graded as follows: 1) abundant pink, or pearly gray, firm tumor nodules scattered diffusely over all peritoneal surfaces, with the major cluster of nodules in the omentum measuring over 10 mm. in diameter (fig. 1), 2) small nodules of the omentum measuring less than 10 mm. in diameter, and 3) no visible nodules. At the time of autopsy all tumors were removed, measured, and representative portions fixed in modified Carnoy's solution or cold, neutral formaldehyde for histologic confirmation.

The cytochemistry of rat-HeLa tumors has been described (*25*), and the cytology of the tumors occurring in rats that had received immune serum will be described separately.

Immune serums.—Rabbit anti-HeLa serum was prepared in 2 adult white rabbits by injecting a total of 4.5 $\times$ 10^6 HeLa cells in 9 injections. HeLa cells were cultivated in medium 199 (90%) and rabbit serum (10%) for at least 6 passages before being used as antigen. The cells were standardized to 1 $\times$ 10^6 cells per ml. of medium 199, mixed with an equal volume of aluminum hydroxide gel adjuvant [9] (*26*), and 1 ml. of the mixture was injected subcutaneously twice a week for 8 doses. Following a 4 week's rest, a booster dose of antigen was injected, and after 2 weeks the rabbits were bled from the heart for antiserum. After bleeding, the serums were pooled, tested for sterility, and stored at 5° C. and −17° C.

Rat anti-HeLa serum was prepared in 30 adult Wistar rats in a similar manner except that the HeLa cells used as antigen were grown in medium 199 (80%) and human serum (20%).

A tuberculin-positive Guernsey cow previously sensitized by injection of old tuberculin was used for the preparation of cow anti-HeLa serum. HeLa cells used as antigen were cultivated in medium 199 (80%) and calf serum (20%) for 6 or more passages prior to use as antigens. A standardized suspension containing 10 $\times$ 10^6 cells per ml. was mixed with an equal volume of aluminum hydroxide gel adjuvant and injected subcutaneously. The injection and bleeding schedules are shown in table 1. After each bleeding, the serum was separated, tested for sterility, and

[9] Aluminum hydroxide gel (Amphojel, Wyeth laboratories) 1 ml. plus 19 ml. of Hank's balanced salt solution.

TABLE 1.—*Immunization and bleeding schedule of tuberculin-positive Guernsey cow injected with HeLa cells grown in calf serum*

Day	HeLa cells inoculated ($\times 10^6$)	Bleeding
0	10	NCS (normal cow serum)
7	20	
11	20	
14	40	
18	40	
28	50	
32	50	
36	50	ICS-1 (immune cow serum-1)
42	50	
56	100	ICS-2
60	100	
73	20	ICS-3
79	100	
85	100	ICS-4
	Total 7.5×10^8	

stored in ampoules at 5° C. and −17° C. The first (NCS) and second bleeding (ICS-1) were used in the *in vitro* experiments as standard negative and positive serum controls. In all animals large granulomata appeared at the sites of antigen injections.

Tests for cytotoxic activity of immune serums.—The cytotoxic activity of the immune serums upon living HeLa cells was measured by noting the inhibition of acid production by varying numbers of HeLa cells when mixed and incubated with varying dilutions of the immune serums. This test is designated the cytotoxic-metabolic inhibition test, hereinafter, the CMI test. Suspensions of freshly harvested HeLa cells were standardized to contain 80,000, 60,000, 40,000, 30,000, 20,000, 15,000, 10,000, 7,500, 5,000, 3,750, 2,500, and 1,875 cells per 0.5 ml. of medium 199 (85%) containing 0.2 percent phenol red and calf serum (15%); 0.5 ml. of each of the 12 cell dilutions was dispensed into separate 13 × 100 mm. tubes. Rabbit, rat, or cow anti-HeLa serums were used unheated or inactivated (60° C. for 30 min.) and 0.1 or 0.25 ml. of various serum dilutions was added to each dilution of HeLa cells. The tubes were stoppered with rubber stoppers or heavy mineral oil (Drakeol, Pennsylvania Refining Co., Butler, Penna.) and incubated at 36 to 37° C. for 14 days. Appropriate controls of normal rabbit, rat, and cow serums were included in each test as well as cell controls, *p*H, mediums, and standard antiserum controls.

The *p*H of all tubes at the start of the test was approximately 7.6 (with the use of 3.0 ml. of 2.8% $NaHCO_3$ per 100 ml. of medium 199). Tubes containing normal control serums changed from red to orange to yellow in all dilutions of HeLa cells containing greater than 1,875 to 2,500 cells. Tubes in which the immune serums prevented the metabolism of HeLa cells gradually changed from red to purple. Cytotoxic endpoints were usually quite sharp and were read as any *p*H greater than 7.0.

Normal, unheated rabbit or guinea-pig serum was used to provide complement and other heat-labile factors, and was diluted to contain 2 to 4 units

of complement in 0.1 ml. as shown by a titration with sensitized sheep cells in the presence of 80,000 HeLa cells per 0.5 ml. For convenience the term complement will be used to refer to the heat-labile factors supplied by normal unheated serum.

HeLa-cell agglutination tests.—A 1 percent suspension of HeLa cells was prepared in saline. Serum dilutions were made in saline, and 0.25 ml. of cells were mixed with 0.5 ml. of each serum dilution, incubated at 37° C. for 30 minutes, overnight at 5° C., and read by pattern.

Complement-fixation tests were carried out by a modified Kolmer technique with overnight fixation at 4° C. using 0.1 ml. of antigen, 0.1 ml. of serum dilution, 0.1 ml. of complement containing 2 exact units, and 0.25 ml. of sensitized sheep red blood cells were added the next day. Antigens were fresh or frozen tissue-culture cell suspensions standardized to contain 2.5×10^6 cells per ml.

Adsorption and viability tests.—Varying numbers of HeLa cells, cynomolgus heart cells (Salk), and human A+ red blood cells were mixed with unheated ICS-3 for 1, 2, 7, and 24 hours at 37° C. Normal cow serum (NCS) mixed and incubated with HeLa cells served as a control. After the appropriate interval, the cells and serum of each test variable were separated. In other tests, 32×10^6 HeLa cells, cynomolugus heart cells (Salk), human conjunctiva and kidney cells (Chang), human intestine cells (Henle), first tissue-culture-passage monkey kidney and rabbit kidney cells, human A+ red blood cells, as well as 1 ml. of packed sediment of human neuroblastoma and Wilms' tumors, were mixed with 10 ml. of ICS-3 for 2 hours at 370° C., separated, and then reabsorbed with the same number of fresh cells for a second 2-hour period. The serums were tested by the CMI test and the complement-fixation test. The cells used for adsorption were tested for viability by two methods: 1) They were resuspended in medium 199 (80%) and human serum (20%), adjusted to 1×10^6, 1×10^5, 1×10^4, 5×10^3, and 2.5×10^3 cells per ml., and 1.0 ml. of each cell dilution was added to each of five 13×100 tubes. These were sealed with heavy mineral oil and incubated at 37° C. for 10 days when *p*H readings were made. 2) Neutral red, 0.2 ml. of a 2 percent solution, was added to 1 ml. of the 1×10^5 dilution of HeLa cells after each interval, and enumerations were made of stained (viable) cells per 100 cells counted.

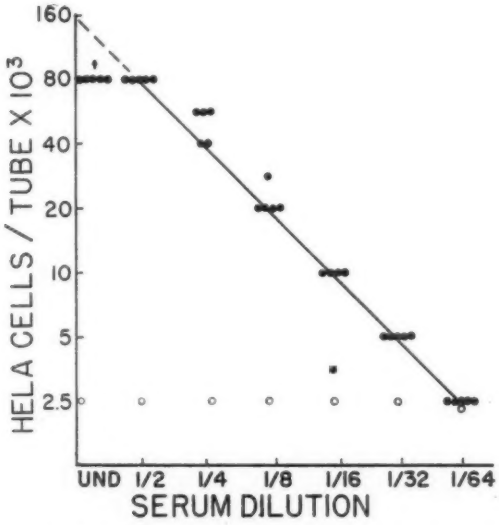
Observations

Effects of Immune Serums on HeLa Cells in Vitro

Cytotoxic metabolic inhibition tests.—Text-figure 1 summarizes 3 CMI tests of ICS-3 against HeLa cells. Undiluted immune serum and serum diluted 1:2 were cytotoxic for 80,000 or more HeLa cells. Immune serum diluted 1:4 was cytotoxic for 40,000 to 60,000 HeLa cells, and in the other serum dilutions a linear relationship existed between the serum dilution and the number of HeLa cells inactivated. The relative potency of the immune serum was expressed as the cytotoxic index (CI) and was

obtained by calculating the antilog (*2*) of the difference between the normal and immune serum endpoints corrected to 1.0 ml. of serum; for example, in text-figure 1, when the curve is projected to the ordinate, 0.1 ml. of undiluted ICS-3 inactivated 1.6×10^5 HeLa cells (log O_2) and NCS inactivated 2.5×10^3 HeLa cells (log 6_2). The antilog (*2*) of the difference = 64, and corrected to 1.0 ml. = 640.

Microscopic examination of the HeLa cells exposed to immune cow serum and complement showed an initial rounding and clumping of the cells followed by lysis or disappearance of all cells in the more concentrated serum dilutions by the end of 24 hours. The effects of immune



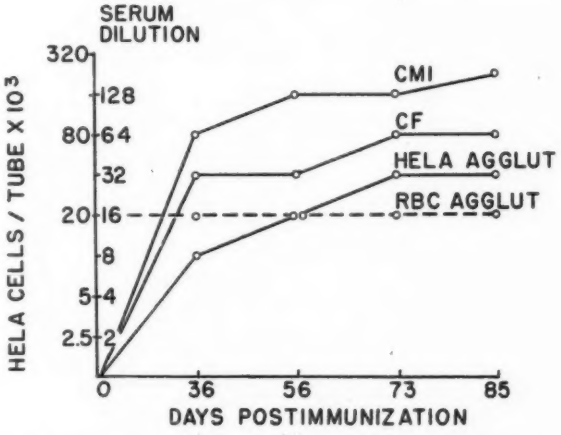
TEXT-FIGURE 1.—Metabolic inhibitory effects *in vitro* of unheated cow anti-HeLa serum (ICS-3) upon HeLa cells. Results of 2 single tests and 1 test run in triplicate. Each point represents the tube containing the greatest number of HeLa cells inhibited by that serum dilution as indicated by *p*H greater than 7.0. *Solid circles* = ICS-3; *open circles* = normal cow serum (NCS). The NCS results were the same for the 3 tests.

rabbit serum upon HeLa cells have been described in detail by Mountain (*27*) and Miller (*28*).

Reproducibility of the CMI test.—The reproducibility of the test suggested by text-figure 1 was further confirmed by repeated tests on the standard cow-immune serum (ICS-1) which was included in each test as positive control, and by triplicate tests on individual serums. All cytotoxicity tests were done by two of the authors (P. G. and R. M.). From these repeated tests the reproducibility of the cytotoxicity tests was found to be within a range of 6 HeLa cell-dilution tubes at each serum dilution. From this it would appear that cytotoxic indices of less than 30 are not significant, 30 to 60 are suggestive, and greater than 60 are significant.

Specificity of cow anti-HeLa cytotoxins.—Nonspecific cytotoxins for HeLa cells were not demonstrated in fresh serum specimens from normal cows, humans, monkeys, rabbits, rats, goats, or guinea pigs when tested in the CMI test.

The specificity of the cow anti-HeLa cytotoxins is suggested in the results summarized in text-figure 2. The results show a gradual increase in cytotoxic, complement-fixing, and HeLa agglutinating activity in the postimmunization bleedings compared to the preimmunization bleeding (NCS). Hemagglutinins for human O−, A−, B−, O+, and A+ red blood cells were present in the normal cow serum and similar titers were present in the postimmunization bleedings. No differences in hemagglutinin titers were noted when the serums were used fresh, inactivated, or inactivated plus 2 units of rabbit or guinea-pig complement. None of



TEXT-FIGURE 2.—The antibody content of successive bleedings from the cow immunized with HeLa cells and tested by the CMI, CF, HeLa cell agglutination, and human red-blood-cell agglutination tests.

the cow serums caused lysis of the human red blood cells when tested fresh or with added guinea-pig complement. Fresh rabbit serum alone caused partial hemolysis of human red blood cells. HeLa cells were derived from a patient whose blood group was 0 positive (*29*), and it is interesting that no antibodies against 0+ antigens were produced in the immunized cow. CMI tests on α-, β-, and γ-globulins and albumin fractions of ICS-4 showed the cytotoxic activity to be in the γ-globulin fraction.[10]

In the CMI test, 10 ml. of ICS-3 was cytotoxic for 16×10^6 HeLa cells. Combined adsorption-viability tests designed to test this relationship were carried out as described under "Materials and Methods." In summary, HeLa cells did adsorb cytotoxins and complement-fixing antibodies from the ICS-3, and a correlation exists between the number

[10] Prepared by Pentex, Inc., Kankakee, Ill., by alcohol fractionation.

of cells used and the amount of cytotoxin adsorbed. The cytotoxic activity and complement-fixing activity were partially adsorbed by cynomolgus heart cells (Salk), human conjunctiva and kidney cells (Chang), human intestine cells (Henle), first tissue-culture-passage monkey kidney cells and rabbit kidney cells, human neuroblastoma emulsion, Wilms' tumor emulsion, and human A+ red blood cells.

The viability of the HeLa cells used in the adsorption tests was measured by subculture and by supravital staining. The results of these tests are summarized in table 2 and suggest that the killing effect of the cytotoxins upon HeLa cells can be measured by either test. The advantage of the supravital-stain method is that results are available within a few minutes and there is good correlation with viability of the cells as shown by subculture.

TABLE 2.—*Viability of HeLa cells used in adsorption of immune cow serum*

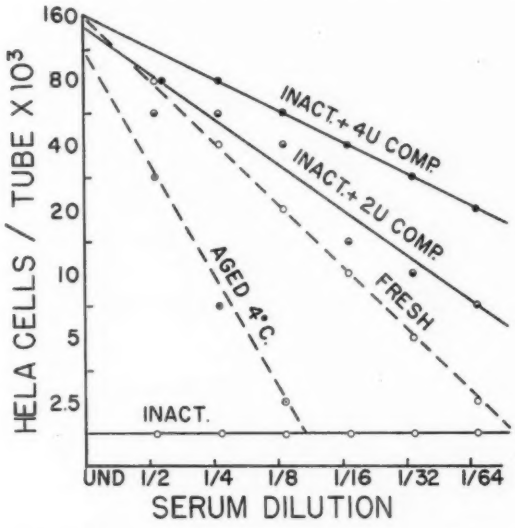
Serum (*10 ml.*)	Number HeLa cells ($\times 10^6$)	Supravital staining on cells after		Cell number required to subculture*	
		1 hour	7 hours	1 hour	7 hours
ICS-3	32	78/100†	3/100	5×10^3	1×10^6
ICS-3	16	8/100	0/100	1×10^5	1×10^6
ICS-3	8	3/100	0/100	1×10^5	$>1 \times 10^6$
ICS-3	4	1/100	0/100	1×10^6	$>1 \times 10^6$
NCS	32	92/100	60/100	2.5×10^3	2.5×10^3

*Least number of cells on subculture that reduced the *p*H of the medium to 7.0 or less.
†Number of cells viable (take neutral red stain)/total cells counted.

TABLE 3.—*Effect of heat upon the cytotoxicity of immune cow serum*

Heat (×30 min.)	Cytotoxic index ICS-3
0 (fresh)	640
50° C.	320
55° C.	40
60° C.	0
65° C.	0

Effect of heat.—As shown in table 3, heating ICS-3 at 60° C. for 30 minutes removed all cytotoxic activity. Text-figure 3 summarizes the effect of adding 2 or 4 units of rabbit complement to heated serum. In these tests inactivated rabbit serum was added to the tubes, which contained no complement, to maintain a constant amount of serum in all tubes. ICS-3 stored at 5° C. for 4 months gradually lost cytotoxic activity but this could be restored by the addition of 2 to 4 units of rabbit complement. Lyophilized guinea-pig serum (Cappel Laboratories, West Chester, Penna.) could not be used as complement. It was toxic to the HeLa cells in the metabolic inhibition test and to monolayer cultures in roller tubes in all dilutions up to 1:200. Fresh guinea-pig serum was nontoxic and has been used for all recent tests.



TEXT-FIGURE 3.—Metabolic inhibitory effects of cow serum (ICS-3) on HeLa cells: when tested fresh and unheated, after storage at 4° C. for 2 months, after heat inactivation at 60° C. for 30 minutes, and after heat inactivation and addition of 2 and 4 units of fresh rabbit complement. Lines drawn connecting the points of each test intersect the ordinate at approximately the same point and form the basis for expressing the cytotoxic effects as the cytotoxic index.

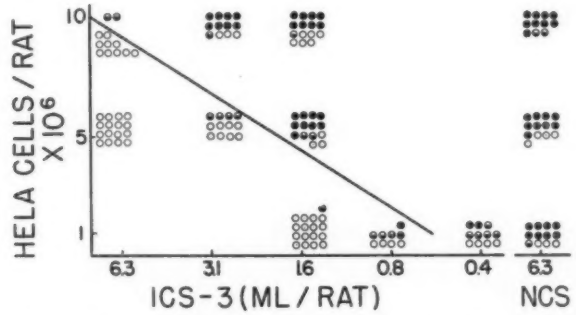
Cytotoxic metabolic inhibition tests on rabbit and rat immune serums.—Rabbit and rat anti-HeLa serums also contained metabolic inhibitory activity against HeLa cells when measured by this test. Unheated rabbit anti-HeLa serum when diluted 1:4 was inhibitory for 80,000 HeLa cells and dilutions of 1:8, 1:16, and 1:32 prevented metabolism of 40,000, 20,000, and 10,000 cells, respectively. Unheated rat anti-HeLa serum undiluted was inhibitory for 47,000 HeLa cells and dilutions of 1:2 and 1:4 prevented metabolism of 27,000 and 10,000 HeLa cells, respectively.

Calculating from the *in vitro* data, 1 ml. of undiluted cow anti-HeLa serum (ICS-3) prevented metabolism of 1.6×10^6 HeLa cells (CI = 640), while 1 ml. of rabbit anti-HeLa serum inhibited 1.92×10^6 HeLa cells (CI = 340), and 1 ml. of rat anti-HeLa serum inhibited 4.7×10^5 HeLa cells (CI = 53).

Effects of immune serums on rat-HeLa tumors.—To determine whether the *in vitro* metabolic inhibitory action of anti-HeLa serums could be demonstrated *in vivo*, a number of preliminary experiments were performed in which rat and rabbit anti-HeLa serums were given in empiric small doses of 0.2 ml. to 2.0 ml. to groups of 15 X-irradiated and cortisone-pretreated rats that had been inoculated intraperitoneally with HeLa cells. No protection was observed.

On the basis of the *in vitro* CMI tests, 5.2 ml. of rabbit serum and 21 ml. of rat serum would be required to kill the 10 million HeLa cells given to

the rats unless the action was greatly potentiated *in vivo*. The availability of large volumes of cow anti-HeLa serum made possible more adequate experiments correlating the *in vitro* effects of this heterologous immune serum upon HeLa cells with the *in vivo* effects and using the same volume of serum per HeLa cell. In the *in vitro* tests, 6.3 ml. of serum would inactivate 1×10^7 cells, 3.1 and 0.8 ml. would inactivate 5×10^6 and 1.25×10^6 HeLa cells, respectively. The 6 experiments designed to test the effect of these volumes of serum *in vivo* are summarized in text-figure 4 and show a close correlation with the *in vitro* results. The unheated cow anti-HeLa serum (ICS-3) was injected intraperitoneally 1 day before the intraperitoneal injection of HeLa cells. In one experiment, 52 rats were given 10×10^6 HeLa cells plus heat-inactivated immune serum in 6.3, 3.1, and 1.6 ml. volumes. The results were similar to those obtained when unheated immune serum was used.



TEXT-FIGURE 4.—Prevention *in vivo* of HeLa tumors in X-irradiated cortisone-treated rats by cow anti-HeLa serum. The serum was injected intraperitoneally 24 hours before the HeLa cells were injected intraperitoneally. Each circle represents 1 rat. *Solid circles* represent an abundant tumor, *half-solid circles* represent small tumors and *open circles* represent no tumor. The diagonal line represents the level of protection predicted from the results of the *in vitro* metabolic inhibition tests, *i.e.*, there should be no tumors to the *left* of the line and there should be tumors to the *right* of the line in numbers equal to the controls shown at the *far right*.

Discussion

Studies in immunology of cancer have been the subject of a recent review (*30*). As noted by Ross (*4*) the importance of continuing the study of anticancer antibodies is twofold. Their value in cancer therapy has never been refuted nor firmly established, but of more immediate importance is the information they may "reveal about cancer cells or processes."

In our experiments, heterologous immune serums were prepared against whole HeLa cells rather than physically or chemically prepared fractions in an effort to retain the antigenic pattern, which may be unique to whole cancer cells (*31*). Studies have been directed at the effects *in vitro* and *in vivo* of circulating cytotoxic antibodies thus produced in the immunized animals (*4*), while no effort has been made to measure cellular immunity or the effects of connective tissue cells or lymphocytes.

The CMI test described by Salk (*17*) is an immunologic technique which our studies show to be reproducible and quantitative. Independent of these studies, Flax (*14*) noted the inability of Ehrlich ascites tumor cells injured by antibody to metabolize glucose while retaining their capacity to utilize succinate. Ross predicted (*4*) that this might be a procedure for the quantitative assay of cytotoxic antibody, and the results reported here support this prediction, though the chemical nature of the metabolic injury has not been defined in our tests.

Specificity of the cytotoxins in the cow immune serum has not been established. However, a number of properties described in this report suggest a specific antibody, as follows: 1) Cytotoxic activity was not present in normal serums from cows, humans, monkeys, rabbits, rats, goats, or guinea pigs. 2) The antibody level increased progressively after repeated exposure of the animal to the HeLa cell antigen. 3) The presence of antibodies was shown by means of cytotoxic metabolic inhibition tests, agglutination tests, and complement-fixation tests. 4) All the tests mentioned were controlled with positive and negative serums and inactivated-complementary system. 5) In the immune cow serum no increase in agglutinins for human red blood cells was demonstrated. 6) The antibody was contained in the γ-globulin fraction. 7) The cytotoxic property was removed by heating to 60° C. and restored by adding fresh normal serum. 8) The antibodies were completely adsorbed by HeLa cells but only partially adsorbed by human type-A-positive red blood cells, other tissue-culture cell lines, human neuroblastoma, and Wilms' tumor homogenates.

Nonspecific cytotoxins for cancer cells have been demonstrated in normal serums by others (*2*, *4*), and Miller (*28*) has reported such cytotoxins for HeLa cells in normal rabbit serum. However, fresh rabbit serum was used as complement in our studies and was not toxic in the concentrations used. Lyophylized guinea-pig serum was found to be toxic to HeLa cells in the CMI test and in monolayer roller-tube cultures. Failure to demonstrate a rise in antibodies to human red blood cells in postimmunization cow serum specimens was unexpected in view of Habel's (*32*) observation of high titers of hemolysins and agglutinins to human red blood cells in the rabbit serums prepared against HeLa cells and other human epithelial cells grown in tissue culture. The rabbit anti-Hela serum produced in our studies also had hemagglutinins (1:512) and hemolysins (1:32) for human red blood cells but these titers were demonstrated only when fortified with guinea-pig complement. This confirms the suggestion of others that the antibody response of an animal to complex cellular antigens is conditioned by his own antigenic structure. Some data suggesting the specificity of cytotoxic antibody against HeLa cells by adsorption studies have been reported by Mountain (*27*) and Björklund (*13*) and are in agreement with the results reported here.

The *in vitro* cytotoxic activity of the cow immune serum was found to be dependent upon complement or other factors present in normal, unheated serum. These factors were not completely destroyed by heating

at 56° C. for 30 minutes but were destroyed by heating at 60° C. for 30 minutes. This may explain the observations of Miller (*28*) and Mountain (*27*) who found rabbit anti-HeLa serum to resist inactivation at 56° C. for 30 minutes.

The rat-HeLa tumor system also offers a quantitative technique for the *in vivo* study of HeLa tumors produced by human cancer cells. Although only approximately 80 to 96 percent of the injected animals develop tumors, these persist for 40 days or more. With the use of this cell line and these two techniques, a remarkable correlation was obtained between *in vitro* metabolic inhibition tests and *in vivo* serum-protection tests. The significance of these data may be questioned because they are based on an artificial cancer system of a human cell in a heterologous, specially pretreated, rodent host. However, there is no natural cancer that can be called "typical" of all malignant growths, and the prospect of a quantitative system (HeLa-rat) offers advantages for exact laboratory experimentation not possible with any other human cancer. Knowledge about any cancer system may be valuable, and the probability of increased knowledge is much greater if one works with a system in which all variables can be reproduced quantitatively.

In vitro CMI tests indicated that 1 ml. of cow anti-HeLa serum (ICS-3) was toxic for 1.6×10^6 HeLa cells. Translating this to *in vivo* tests, rats injected with 10×10^6 HeLa cells should have been protected by 6.3 ml. of serum, and this amount proved to be protective while smaller volumes of serum were not significantly different from controls. The same range of correlation was obtained with 5×10^6 and 1×10^6 injected HeLa cells in rat-protection tests with lesser amounts of immune serum calculated by results of *in vitro* tests. *In vivo* protection tests with inactivated serum gave essentially the same results as unheated immune serum, which made it appear that the rat supplied the complement or other heat-labile factors required and that the HeLa cells were not inactivated by the irradiation and cortisone treatment of the rat.

Previous authors have noted the difficulty with which anti-cancer γ-globulin crosses the capillary membrane in solid tumors (*3*, *10*, *11*, *33*). Others have demonstrated the efficacy of such antibodies in the treatment of ascites tumors when injected intraperitoneally (*3*, *33*), thereby avoiding the necessity of passage across capillary membranes. In our studies the close correlation between the test tube and the treated rat would suggest that either the antibody remained in the peritoneal cavity or that it exchanged freely across the capillary wall. There is no proof that the γ-globulin leaves the peritoneal cavity, and studies designed to answer this question are in progress.

These data suggest that heterologous antiserum can prevent at least one type of experimental human tumor and that new quantitative *in vitro* and *in vivo* tests are now available for exploring the mechanism of tumor rejection.

The authors wish to acknowledge the aid of Dr. P. T. Gilbert and Dr. J. T. Harkeson, of the X-Ray Department, Cooper Hospital, Camden, New Jersey.

References

(1) Hauschka, T. S.: Immunologic aspects of cancer: *A review.* Cancer Res. 12: 615–633, 1952.

(2) Mohos, S. C., and Kidd, J. G.: Effects of various immune rabbit serums on the cells of several transplanted mouse lymphomas in vitro and in vivo. J. Exper. Med. 105: 233–263, 1957.

(3) Wissler, R. W., and Flax, M. H.: Cytotoxic effects of antitumor serum. Ann. New York Acad. Sc. 69: 773–794, 1957.

(4) Ross, J. D.: Cytotoxins and cytotoxic antibodies. Ann. New York Acad. Sc. 69: 795–800, 1957.

(5) Nettleship, A.: Regression produced in the Murphy lymphosarcoma by the injection of heterologous antibodies. Am. J. Path. 21: 527–543, 1945.

(6) Nungester, W. J., and Fisher, H.: The inactivation *in vivo* of mouse lymphosarcoma 6C3HED by antibodies produced in a foreign host species. Cancer Res. 14: 284–288, 1954.

(7) Imagawa, D. T., Syverton, J. T., and Bittner, J. J.: The cytotoxicity of serum for mouse mammary cancer cells. I. The effects of admixture *in vitro* upon homoiotransplantability. Cancer Res. 14: 1–7, 1954.

(8) Horn, E. C.: Ascites tumor development. II. Cytotoxicity of various antisera prepared against Ehrlich ascites tumor cell components. Cancer Res. 16: 595–599, 1956.

(9) Björklund, B.: Antigenicity of malignant and normal human tissues by gel diffusion techniques. Internat. Arch. Allergy 8: 179–192, 1956.

(10) Pressman, D., and Korngold, L.: The in vivo localization of anti-Wagner-osteogenic-sarcoma antibodies. Cancer 6: 619–623, 1954.

(11) Korngold, L., and Pressman, D.: The localization of antilymphosarcoma antibodies in the Murphy lymphosarcoma of the rat. Cancer Res. 14: 96–99, 1954.

(12) Björklund, B., Graham, J. B., and Graham, R. M.: Effect of horse anti-human cancer serum on malignant and normal human cells: Preliminary report. Internat. Arch. Allergy 10: 56–64, 1957.

(13) Björklund, B., and Björklund, V.: Antigenicity of pooled human malignant and normal tissues by cyto-immunological technique: Presence of an insoluble, heat-labile tumor antigen. Internat. Arch. Allergy 10: 153–184, 1957.

(14) Flax, M. H.: The action of anti-Ehrlich ascites tumor antibody. Cancer Res. 16: 774–783, 1956.

(15) Gey, G. O., Coffman, W. D., and Kubicek, M. T.: Tissue culture studies of the proliferative capacity of cervical carcinoma and normal epithelium. Cancer Res. 12: 264–265, 1952.

(16) Scherer, W. F., Syverton, J. T., and Gey, G. O.: Studies on the propagation in vitro of poliomyelitis viruses. IV. Viral multiplication in a stable strain of human malignant epithelial cells (strain HeLa) derived from an epidermoid carcinoma of the cervix. J. Exper. Med. 97: 695–710, 1953.

(17) Salk, J. E., and Ward, E. N.: Some characteristics of a continuously propagating cell derived from monkey heart tissue. Science 126: 1338–1339, 1957.

(18) Toolan, H. W.: Successful subcutaneous growth and transplantation of human tumors in X-irradiated laboratory animals. Proc. Soc. Exper. Biol. & Med. 77: 572–578, 1951.

(19) ———: Transplantable human neoplasms maintained in cortisone-treated laboratory animals: H. S. #1; H.Ep. #1; H.Ep. #2; H.Ep. #3, and H.Emb. Rh #1. Cancer Res. 14: 660–666, 1954.

(20) Suskind, R. G., Huebner, R. J., Rowe, W. P., and Love, R.: Viral agents oncolytic for human tumors in heterologous host. Oncolytic effect of Coxsackie B viruses. Proc. Soc. Exper. Biol. & Med. 94: 309–318, 1957.

(*21*) CORIELL, L. L., MCALLISTER, R. M., and WAGNER, B. M.: Criteria for determining malignancy in tissue culture cell lines in the Albino rat. Special Publications of the New York Acad. of Sc. 5: 341–350, 1957.

(*22*) MORGAN, J. F., MORTON, H. J., and PARKER, R. C.: Nutrition of animal cells in tissue culture: initial studies on a synthetic medium. Proc. Soc. Exper. Biol. & Med. 73: 1–8, 1950.

(*23*) MCALLISTER, R. M., and CORIELL, L. L.: Cultivation of human epithelial cells in tissue culture. Proc. Soc. Exper. Biol. & Med. 91: 389–394, 1956.

(*24*) MELNICK, J. L., and OPTON, E. M.: Assay of poliomyelitis neutralizing antibody in disposable plastic panels. Bull. World Health Organ 14: 129–146, 1956.

(*25*) LOVE, R., SUSKIND, R. G., and HUEBNER, R. J.: Regression of HeLa tumors in cortisone-treated irradiated rats following infection with Coxsackie B3 virus. A histological and cytochemical study. J. Nat. Cancer Inst. 19: 49–77, 1957.

(*26*) COLIO, L. G., CRILEY, B. R., and CORIELL, L. L.: Treatment of poliomyelitis in monkeys with hyperimmune rabbit gamma globulin. J. Immunol. 80: 278–281, 1958.

(*27*) MOUNTAIN, I. M.: Cytopathogenic effect of antiserum to human malignant epithelial cells (strain HeLa) on HeLa cell culture. J. Immunol. 75: 478–484, 1955.

(*28*) MILLER, D. G., and HSU, T. C.: The action of cytotoxic antisera on the HeLa strain of human carcinoma. Cancer Res. 16: 306–312, 1957.

(*29*) GEY, G. O.: Personal communication.

(*30*) SYVERTON, J. T.: Consulting Editor. Immunology and Cancer. Ann. New York Acad. Sc. 69: 525–856, 1957.

(*31*) COMAN, D. R., and ANDERSON, T. F.: A structural difference between the surfaces of normal and of carinomatous epidermal cells. Cancer Res. 15: 541–543, 1955.

(*32*) HABEL, K., HORNIBROOK, J. W., and GREGG, N. C.: Cytotoxic effects of antisera against human epithelial cells grown in tissue culture. Ann. New York Acad. Sc. 69: 801–803, 1957.

(*33*) WISSLER, R. W., BARKER, P. A., FLAX, M. H., LAVIA, M. F., and TALMAGE, D. W.: A study of the preparation, localization, and effects of antitumor antibodies labeled with I^{131}. Cancer Res. 16: 761–773, 1956.

PLATE 65

FIGURE 1.—X-irradiated and cortisone-treated rat with intraperitoneal tumors 21 days after the injection of 10 million HeLa cells from tissue culture. The skin and abdominal wall were reflected to show the tumor implants.

468155—58——12

The Carcinogenicity for Mouse Skin and the Aromatic Hydrocarbon Content of Cigarette-Smoke Condensates[1,2]

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Summary

The carcinogenic activities of 2 cigarette tars, 1 prepared at New York University and 1 at Sloan-Kettering Institute, were compared by skin painting on mice under identical conditions. Cancers developed in 18 percent of the animals painted with SKI tar and in 8 percent of the animals painted with NYU tar. Chemical analysis of the 2 tars for 3 polynuclear aromatic hydrocarbons showed that the SKI tar possesses each of these compounds in concentrations 3 to 4 times as high as the NYU tar. **J. Nat. Cancer Inst. 21: 557–561, 1958.**

Although there have been many reports of epidemiologic studies, both prospective and retrospective, indicating an association between cigarette smoking and pulmonary cancer, there have been few reports of the experimental production of tumors by cigarette tar. Such studies were reviewed by Wynder, Graham, and Croninger in their report on the application of cigarette-smoke condensate to the skin of mice (*1*). They reported that papillomas were obtained in 59 percent and carcinomas in 44 percent of 81 mice receiving cigarette-smoke condensate; the mean time of appearance of the papillomas was 56 weeks and of the carcinomas 71 weeks. As part of a combined chemical and biologic study of cigarette-smoke condensate (tobacco tar) in our laboratories, painting experiments were carried out with tobacco tars prepared in these studies (*2*). A much lower yield of tumors was obtained in this work than in that reported by Wynder *et al.* in their work cited.

A number of factors seemed to be significant in these variant results. Among them were differences not only in methods of tar production but also in the manner of application, dosage, and strain of animals used. It

[1] Received for publication March 24, 1958.
[2] Aided by a grant from the American Cancer Society, Inc.
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[4] The cooperation of Dr. E. L. Wynder, of the Sloan-Kettering Institute, in supplying the SKI tar is gratefully acknowledged.

was therefore decided to undertake a direct comparison of tar prepared by Wynder at Sloan-Kettering Institute with tar prepared at New York University. All factors other than the nature of the tar, *e.g.*, the method and frequency of painting, and strain of mice, were to be the same for the 2 test groups and similar to those reported by Wynder, Graham, and Croninger (*1*). Simultaneously, a comparative chemical analysis of the 2 tars was to be undertaken to determine the concentrations of selected aromatic hydrocarbons.

Experiments and Results

The cigarette tars designated "NYU" were prepared as described earlier (*2*), while those furnished by Wynder and designated "SKI" were prepared at Sloan-Kettering Institute in a manner like that described in (*1*).

Skin-painting experiments.—Fifty mice, female Swiss-Millerton about 8 weeks old, were used in each group. The mice were painted 3 times weekly with a #5 camel's-hair brush, which delivered approximately 100 mg. of solution per application. The cigarette tars were used as 50 percent solutions in acetone. The entire back of the animal was painted, and the hair clipped when necessary.

The first papilloma in the SKI group appeared by 150 days; the first cancer by 300 days. The first papilloma appeared in the NYU tar group by 140 days; the first cancer by 150 days. At 24 months there were no survivors, and a total of 9 papillomas and 9 cancers had appeared in the SKI group, and 6 papillomas and 4 cancers in the NYU group (table 1). One papilloma regressed in the NYU group, 2 in the SKI group.

Because of the high initial mortality in the group on SKI tar (13 of 50 mice within 90 days), 15 additional mice were started. However, all the mice in the additional group were dead by 360 days; 1 papilloma and 1 cancer were observed. These animals are not included in table 1.

TABLE 1.—*Number of papillomas and cancers in Swiss-Millerton mice painted with cigarette-smoke condensates*

Months	NYU tar			SKI tar		
	Number of survivors	Tumors Cumulative total		Number of survivors	Tumors Cumulative total	
		Papillomas	Cancers		Papillomas	Cancers
0	50	0	0	50	0	0
4	46	1	0	37	0	0
5	46	1	1	37	1	0
6	46	1	1	37	4	0
10	40	4	1	32	6	2
12	28	5	1	24	9	5
18	8	5	4	0	9*	9
23	3	6†	4	—	—	—

*Two papillomas regressed.
†One papilloma regressed.

All tumor designations were based on histologic examinations.

Chemical analysis of the whole tars.—The procedure previously described (*3*) was used with minor modifications. Both tar samples were subjected to the same fractionation and identification procedures.

(a) Isolation of the neutral fraction: The volatiles, acids, and bases were removed (*3*) from 100 gm. of each tar. The NYU tar gave 29.5 gm. and the SKI tar 36.5 gm. of neutral tar.

(b) Alumina chromatography of the neutral fraction: The neutral fractions were chromatographed on acid-washed alumina and the fractions shown in table 2 were obtained. Fraction II was evaporated in vacuum, made up to 100 ml. (solution 1) in benzene, and 25 ml. of this solution was rechromatographed on activated alumina. The benzene eluate from this chromatography was evaporated to a small volume in vacuum and made up to 10 ml. in benzene (solution 2). Another 25 ml. portion of solution 1 was rechromatographed on activated alumina and the eluate obtained with 75 percent benzene-25 percent petroleum ether (b.p. 30 to 60° C.) evaporated and made up to 5 ml. (solution 3) in benzene.

TABLE 2.—*Chromatography of neutral fractions on alumina*

Fraction number	Solvent	Volume of solvent (*liters*)	
		NYU tar	SKI tar
I	Petroleum ether (b.p. 30–60° C.)	2. 3	2. 5
II	Benzene	4. 2	6. 0
III	Acetic acid	0. 5	0. 5

(c) Paper chromatography of solutions 2 and 3: The procedure for the paper chromatography has been described in detail (*3*). Portions (800λ) of both of the solutions 2 were chromatographed on paper and the areas corresponding in R_F values to those of benzo[*a*]pyrene and benzo[*e*]pyrene were eluted and their hydrocarbon concentrations determined from ultraviolet absorption spectrums. In the same manner, the pyrene concentrations were determined in each solution 3. The results are given in table 3.

(d) Weight loss determinations: One gram samples of the whole tars were dried in an oven at 105° C. for 2 hours and the loss of weight was

TABLE 3.—*Yields of polynuclear hydrocarbons from SKI and NYU whole tars*

Compound	Yield, SKI tar		Yield, NYU tar		Ratio SKI/NYU
	μg./100 cigarettes*	μg./100 gm. tar	μg./100 cigarettes†	μg./100 gm. tar	
Benzo[*a*]pyrene	4. 3	100	0. 7	30	3. 3/1
Benzo[*e*]pyrene	1. 8	41. 8	0. 3	12. 4	3. 3/1
Pyrene	43. 7	1017	6. 1	265	3. 8/1

*Based on reported yield of 43 mg. tar/100 cigarettes.
†Based on reported yield of 23 mg. tar/100 cigarettes.

determined. The NYU tar used for the chemical analysis showed a 50.3 percent weight loss, while the SKI tar showed a 41.5 percent loss.

Discussion

The results listed in table 1 clearly show that both the SKI and NYU cigarette tars are definitely, though not strongly, carcinogenic, and thus confirm earlier positive reports. In 1 of the 2 earlier and essentially negative painting experiments carried out in this laboratory with whole tar, the dosage was limited to a low level because of the toxicity; in the other preliminary study the dosage was considerably higher and the animals did not survive into the period where tumor induction might be observed. In the experiments reported here, the toxicity, though high, still permitted sufficient survival for the carcinogenicity of the tars to become apparent.

The greater activity of the SKI tar was evident in higher toxicity as well as in larger tumor yield. Thus the mice in the SKI group showed a much higher mortality in the early months; 13 were dead by the 4th month, while only 4 of the NYU group had died in the same period. By 18 months there were no survivors in the SKI group but there were 8 in the NYU group.

The observed higher biologic activity of the SKI tars clearly is related to the fact that the tar is more concentrated; the greater concentration of the SKI tar is evident both in its greater nonvolatile content and higher concentration of polynuclear aromatic compounds. The higher potency of the SKI tar may be traceable to differences in the method of preparation. The SKI tar was collected in Dry Ice-acetone traps and concentrated by prolonged evaporation in open evaporating dishes under electric fans, while the NYU tar was collected in liquid-air traps and used without further treatment.

The 3 aromatic hydrocarbons chosen for analysis represent a potent carcinogen (benzo[*a*]pyrene), a weak one (benzo[*e*]pyrene), and a related inactive hydrocarbon (pyrene). Their relative concentrations were about the same in the 2 tars, each being 3 to 4 times higher in the SKI tar. This uniformity of concentration may be of some additional interest when it is noted that the smoking patterns used in the preparation of the 2 tars were quite different; the SKI tar was produced by a 30-second puff cycle in contrast to the 1-minute cycle with the NYU tar. The duration of puff was 2 seconds in both cases.

The presence of the carcinogenic polynuclear aromatic compounds and the fact that they occur in about the same ratio as their biologic activity cannot, of course, be taken as a demonstration that all the carcinogenicity stems from this type of compound. It can be estimated that, relative to a pure solution of benzo[*a*]pyrene, the carcinogenic activity of the NYU tar is about equivalent to that of a 15 p.p.m. (15 μg./gm.) solution of the hydrocarbon and, correspondingly, the SKI tar to a 30 p.p.m. solution (*4*). However, in our analysis only about 0.15 p.p.m. benzo[*a*]pyrene (50% solution of 0.3 p.p.m.) could be found in the solution of NYU tar

used in painting the mice, and, correspondingly, 0.5 p.p.m. for the SKI tar. Thus the concentrations found are, respectively, 100 and 60 times lower than might be anticipated from the biologic activity of the 2 tars. We have identified a number of carcinogenic polynuclear aromatic compounds in cigarette tar (*3*, *5*) other than benzo[*a*]pyrene, and these, and others still unidentified, contribute in all likelihood to the observed biologic activity. However, these may still be inadequate to explain the observed biologic activity and it may be that either strong potentiators or carcinogenic compounds of other types, or both, are present and contribute to the carcinogenicity of the cigarette tars for mouse skin.

References

(*1*) WYNDER, E. L., GRAHAM, E. A., and CRONINGER, A. B.: The experimental production of carcinoma with cigarette tar. Cancer Res. 13: 855–864, 1953.

(*2*) KOSAK, A. I., SWINEHART, J. S., and TABER, D.: The components of cigarette smoke. I. Preliminary studies. J. Nat. Cancer Inst. 17: 375–389, 1956.

(*3*) VAN DUUREN, B. L.: Identification of some polynuclear hydrocarbons in cigarette-smoke condensate. J. Nat. Cancer Inst. 21: 1–16, 1958.

(*4*) WYNDER, E. L., FRITZ, L., and FURTH, N.: Effect of concentration of benzopyrene in skin carcinogenesis. J. Nat. Cancer Inst. 19: 361–370, 1957.

(*5*) VAN DUUREN, B. L.: The polynuclear aromatic hydrocarbons in cigarette-smoke condensate. II. J. Nat. Cancer Inst. In press.

Cytotoxic Antibodies in the Homograft Reaction [1]

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Summary

An analysis was made of the cytotoxic isoantibodies that can be demonstrated *in vitro* by the addition of complement, using, mainly, the strain specific tumors 6C3HED and DBA, and the homotransplantable MC1M, MC1A, 6C3HED-ICR and DBA-ICR. Homografts of the strain-specific tumors can elicit production of the cytotoxic antibodies. The cytotoxins produced by 6C3HED are effective against 6C3HED but not against MC1M, though homografts of MC1M and of normal C3H tissues can elicit cytotoxins active against both MC1M and 6C3HED. Cells of 6C3HED therefore possess one toxinogen, C', shared by MC1M and normal C3H tissues, whereas the latter possess a second toxinogen, M', which is not present in 6C3HED. This was confirmed by absorptive experiments. Homografts of MC1A, 6C3HED-ICR, and DBA-ICR, did not elicit production of the cytotoxic antibodies. These tumors were also not affected by the cytotoxins produced by 6C3HED, MC1M, or DBA. DBA-ICR lost the toxinogens of DBA. Cells of MC1A and 6C3HED-ICR lost the C' antigen of 6C3HED and the M' of MC1M. *In vitro* absorption tests demonstrated that MC1A possesses a nontoxinogenic antigen, A', that is related to an MC1M toxinogen. MC1M has the antigenic constitution, C', M', A', with C' and A' forming a complex antigen that produces a complex antibody molecule. It was shown that in the absence of added complement, these cytotoxic antibodies do not produce a destruction of the tumor cells *in vivo*. Thus they seem to play no direct role in the homograft reaction. **J. Nat. Cancer Inst. 21: 563–578, 1958.**

The immunological mechanism responsible for the destruction of tissue homografts is still an open question, and with the exception of cases of leukemia (*1*, *2*) there has been so far no evidence that circulating antibodies play a decisive role in this reaction. The demonstration by Gorer and O'Gorman (*3*) of the cytotoxic activity of isoantibodies in mice by the *in vitro* addition of complement suggested that a study of these anti-

[1] Received for publication March 24, 1958.

468155—58——13

bodies may shed new light on the mechanism responsible for homograft destruction. In the present study an analysis has therefore been made: 1) of the distribution of the antigens responsible for the production of these cytotoxic antibodies in tumors with different degrees of homotransplantability; 2) the relationship between the presence of these antigens and homotransplantability; 3) the nature of these cytotoxic antibodies; and 4) their role in the destruction of tissue homografts.

Materials and Methods

The ascites tumors used in the present study were the 6C3HED, MC1M, MC1A, and 6C3HED-ICR tumors that originated in C3H mice, and the DBA and DBA-ICR tumors that originated in DBA/2 mice. Of these tumors, which have been further described in our previous studies (*4–6*), 6C3HED and DBA are strain specific, and MC1M gives successful intraperitoneal homografts, whereas MC1A, 6C3HED-ICR, and DBA-ICR give successful homografts after both intraperitoneal and intramuscular inoculation. The solid tumors all originated in C3H mice and, of these, R4, R12, and W1 are strain specific, whereas R2A and R2B are homotransplantable (*7*). As in our previous studies, the C3H, C57BL, and DBA/2 mice are from brother × sister matings derived from inbred lines originally obtained from Dr. W. E. Heston of the National Cancer Institute (C3H and C57BL), and from the Roscoe B. Jackson Memorial Laboratory (DBA/2). The Swiss mice used have been brother × sister mated in our laboratory for at least 20 generations.

The method of detecting cytotoxic antibodies was that of Gorer and O'Gorman (*3*). This is based on the assumption that 1) the cytotoxic effect of the antibodies is similar to the lytic effect of bactericidal antibodies in their requirement for complement, and 2) mouse antiserum *in vitro* is anticomplementary. Therefore in order to carry out the cytotoxic antigen-antibody reaction, guinea-pig complement should be added to the *in vitro* cell-antiserum system.

In nearly all the tests for these cytotoxic antibodies, hyperimmune serum has been used. When testing the susceptibility of the ascites tumors to the cytotoxins, ascites cells were drawn out into a solution of 4 percent citrate, and then washed several times in Tyrode's solution to remove all the intercellular ascitic fluid. A comparison of saline and citrate showed that the citrate did not interfere with the reaction. After washing, the tumor cells were suspended in 1:5 normal mouse serum in Tyrode's solution. Unless otherwise stated, the antiserum used was diluted 1:1 in Tyrode's solution; 0.1 cc. of cell suspension was mixed with 0.1 cc. of antiserum, and to this 0.06 cc. or 0.08 cc. of complement (Dehydrated Complement, Texas Biological Labs.) was usually added. The mixture was incubated for 1 hour at 37° C., and 2 cc. of trypan blue (0.05% in Tyrode's solution) was then added to each test tube. Control tests for each cell sample that was examined for cytotoxins were similarly made by incubating the cells with complement and normal serum. The

percent of stained cells was determined by counting at least 200 cells in a hemocytometer. Absorptions were carried out at 37° C.

Hemagglutinins were determined by the dextran-human serum method of Gorer and Mikulska (*8*).

Experiments

The Production of Cytotoxic Antibodies by Homografts of Tumors Originating in C3H Mice

The *in vitro* cytotoxic activity of antiserums produced by homografts of tumors with different degrees of homotransplantability was first studied with the following tumors of C3H origin: 6C3HED, 6C3HED-ICR, MC1M, and MC1A. Each experimental group of antiserum producers consisted of 6 to 8 animals of either C57BL or Swiss mice, and hyperimmune serums were produced by 3 to 5 inoculations of ascites cells. Since MC1M and MC1A intraperitoneal homografts grew progressively in nonimmunized hosts, the tumors in these groups were washed out after the first inoculation (*5*). The transplantation immunity produced by one graft of MC1M and MC1A prevented the progressive growth of the second tumor inoculation (*5*) and no further washings were required. However, one intraperitoneal inoculation of 6C3HED-ICR did not always prevent the growth of a second graft of this tumor (*6*), so that in this case each cell inoculation was followed by tumor removal prior to the following inoculation. The serums of all animals in each group were pooled and the isoantibody level of the antiserums was routinely determined by hemagglutinin titrations. The serums tested for cytotoxic activity were always of a high agglutinin titer (not lower than 2^9). Antiserum was also produced against normal C3H liver and spleen by 4 to 5 intraperitoneal inoculations of the minced tissues.

The susceptibility of the tumor cells to the cytotoxic activity of these antiserums was studied from two points of view. An analysis was made 1) of the capacity of the tumor cells to elicit cytotoxic antibodies against cells from the same tumor and 2) of the susceptibility of the cells of each tumor to the cytotoxic antibodies produced by the other C3H-originated tumors and to those produced by homografts of normal C3H tissues. The results, summarized in table 1, show the following immunological properties of normal tissues and cells of the different tumors:

Normal tissues.—Homografts of C3H liver and spleen in Swiss mice elicited antibodies that showed cytotoxic activity against 6C3HED and against MC1M cells. The lower complement concentration seems to have activated a more intense cytotoxicity against 6C3HED than against MC1M. No cytotoxic effect was found against cells of MC1A and 6C3HED-ICR.

6C3HED.—Homografts of 6C3HED in C57BL hosts have produced cytotoxins active against 6C3HED cells, but not against MC1M, MC1A, and 6C3HED-ICR. The cells of these three tumors were therefore completely nonsusceptible to anti-6C3HED antiserum.

MC1M.—Homografts of MC1M in C57BL mice produced antibodies showing cytotoxic activity against MC1M and 6C3HED but not against MC1A or 6C3HED-ICR cells. It should be noted that samples of normal MC1M cells, without the application of antiserum, showed a greater incidence of affected cells revealed by the trypan-blue method than cells of the other tumors. But this did not seem to be due to a special susceptibility to complement since similar frequencies of stained cells were also found without the complement treatment.

MC1A.—Homografts of MC1A in C57BL mice produced antiserum that had no cytotoxic effect on any of the tumors tested. The apparent high percentage of stained cells in the 0.06 cc. complement test is not significant since the controls in this group also showed a high incidence of such cells.

6C3HED-ICR.—Homografts of 6C3HED-ICR in C57BL mice were similar to MC1A in producing antiserums that showed no cytotoxic activity against any of the tumors tested.

TABLE 1.—*Cytotoxic activity of antinormal C3H liver and spleen, 6C3HED, MC1M, MC1A, and 6C3HED-ICR*

Serum*	Cells tested				Hemagglutinin titer
	6C3HED†	MC1M	MC1A	6C3HED-ICR‡	
Antinormal-C3H ^{XXA}	92	41	9	4	$>2^{14}$
Control	16	21	7	5	
Antinormal-C3H ^{XXC}	68	61	9	—	$>2^{14}$
Control	15	17	6	—	
Anti-6C3HED ^{XB}	87	11	7	8	$>2^{13}$
Control	7	13	7	7	
Anti-6C3HED ^{XB}	61	16	3	6	2^{11}
Control	. 3	18	4	4	
Anti-MC1M ^{XA}	69	42	10	4	2^{12}
Control	17	21	9	5	
Anti-MC1M ^{XA}	65	64	46	10	$>2^{14}$
Control	24	12	39	16	
Anti-MC1M ^{XB}	71	96	31	—	—
Control	24	30	29	—	
Anti MC1A ^{XB}	10	20	11	11	$>2^{10}$
Control	12	25	12	9	
Anti-MC1A ^{XA}	40	11	25	19	2^{9}
Control	33	9	27	16	
Anti-6C3HED-ICR ^{XB}	14	31	4	5	$>2^{10}$
Control	12	30	12	9	
Anti-6C3HED-ICR ^{XB}	5	11	4	4	2^{10}
Control	4	18	3	5	

*X = Antiserum produced in C57BL mice; XX = antiserum produced in Swiss mice; A = 0.06 cc; B = 0.08 cc; and C = 0.1 cc. complement. All antiserums used at a dilution of 1 in 2.

†Percent of stained cells.

‡— = Not tested.

It has thus been found that grafts of the nonhomotransplantable tissues such as normal liver and spleen, the 6C3HED tumor, and the homotransplantable MC1M elicit antibodies with a cytotoxic effect when activated *in vitro* by complement and tested against themselves. On the other hand the homotransplantable tumors MC1A and 6C3HED-ICR produce antiserums that are neither cytotoxic against themselves nor do they reveal any cytotoxicity against the other C3H originated tumors. Furthermore, the cells of these two homotransplantable tumors are completely nonsusceptible to the cytotoxic isoantibodies produced by the normal tissue grafts or by 6C3HED and MC1M. It can therefore be concluded that the cells of the homotransplantable MC1A and 6C3HED-ICR do not contain the antigen(s) responsible for the production of the cytotoxins. But the distribution of these toxinogens, *i.e.*, of the antigens eliciting cytotoxic antibodies, is not correlated with the degree of homotransplantability—6C3HED is a strain-specific tumor, whereas MC1M is not. Yet homografts of 6C3HED elicited cytotoxic antibodies that were active only against 6C3HED cells, whereas homografts of MC1M produced cytotoxins effective against both 6C3HED and MC1M. MC1M is thus in this respect similar to normal somatic tissues.

These differences between the properties of 6C3HED and those of MC1M and normal tissues can be explained by the assumption that cells of MC1M and those of normal liver and spleen contain two different antigens, only one of which is shared by the 6C3HED cells. It may be assumed that a complete cytotoxic effect on MC1M is obtained only when the 2 antigens of the tumor cells are affected. Anti-MC1M and anti-liver and -spleen therefore acted cytotoxically on MC1M cells, whereas anti-6C3HED was not sufficient to produce a cytotoxic effect on MC1M cells. On the other hand, anti-MC1M and anti-liver and-spleen having the one antigen of 6C3HED should, according to this assumption, be cytotoxic to 6C3HED cells, as they were experimentally shown to be.

In Vitro *Absorption of Cytotoxic Antibodies by Tumors Originating in C3H Mice*

The conclusions on the toxinogen distribution of tumor cells derived from the previous experiments were checked by *in vitro* absorption tests. The antiserums tested for the absorption of cytotoxic antibodies by tumor cells were C57BL anti-6C3HED and C57BL anti-MC1M. Each antiserum was divided into 5 samples, of which one each was absorbed by 6C3HED, MC1M, MC1A, 6C3HED-ICR, and one was left unabsorbed as a control. Each sample was absorbed twice with tumor cells and the absorbed antiserums were tested for cytotoxins. Anti-6C3HED was tested against 6C3HED cells and anti-MC1M against 6C3HED and MC1M cells.

The results given in table 2 show that 6C3HED and MC1M removed all cytotoxic activity from anti-6C3HED, whereas MC1A and 6C3HED-ICR did not absorb the cytotoxicity against 6C3HED. These results

are consistent with those obtained by the direct study of the activity of these antiserums when cross tested with cells of the various tumors. The 6C3HED cells elicited cytotoxic isoantibodies and, accordingly, removed them by absorption. MC1M was shown to share one antigen with 6C3HED and was therefore expected to absorb the anti-6C3HED activity, as shown experimentally, though anti-6C3HED did not produce a cytotoxic effect on MC1M cells. On the other hand, MC1A and 6C3HED-ICR, which do not produce cytotoxins to 6C3HED cells, did not absorb them from anti-6C3HED serum. These results support the conclusions drawn from the previous experiments that MC1M shares with 6C3HED the antigen responsible for the cytotoxicity of anti-6C3HED, whereas MC1A and 6C3HED-ICR seem to have lost this antigen.

TABLE 2.—In vitro *absorption of cytotoxic antibodies by cells of 6C3HED, MC1M, MC1A, and 6C3HED-ICR**

Serum	Cells tested	Unabsorbed‡	Absorbed with†			
			6C3HED	MC1M	MC1A	6C3HED-ICR
Anti-6C3HED..	6C3HED	87	10	12	72	94
Control.....	6C3HED	7	7	7	7	7
Anti-MC1M....	6C3HED	69	12	17	8	47
Control.....	6C3HED	17	17	17	17	17
Anti-MC1M....	MC1M	42	27	18	32	37
Control.....	MC1M	21	21	21	21	21

*Antiserums produced in C57BL mice and used at a dilution of 1 in 2 with 0.08 cc. complement.
†Two absorptions for 1 hour at 37° C.
‡Percent of stained cells.

Similar experiments were carried out with anti-MC1M antiserums but here each absorbed sample was tested both against 6C3HED and MC1M cells. It was found (table 2) that absorption with 6C3HED, MC1M, and MC1A removed completely the cytotoxic activity against 6C3HED. The absorption of the cytotoxins by 6C3HED and MC1M is consistent with the evidence on the distribution of the toxinogens obtained by the direct tests. On the other hand, the removal of cytotoxic activity by MC1A was unexpected and indicated that some relationship exists between a nontoxinogenic antigen of MC1A and the toxinogen of 6C3HED. When these absorbed antiserums were tested against MC1M cells, 6C3HED and MC1M removed, as expected, all the anti-MC1M activity. MC1A also showed some absorption of the anti-MC1M activity, though the degree of absorption here was lower than that demonstrated when tested against 6C3HED cells. These results indicate that some cross reaction exists between antigen(s) of the MC1A cells and antibodies produced by MC1M homografts. A possible immunological interpretation of this rather unexpected cross reaction will be discussed later.

Another set of absorption experiments was carried out with a series of strain C3H-originated solid tumors. Anti-C3H liver was produced

in Swiss mice, and the tumors tested were the following: R2A, R2B, R4, R12, and W1, the first two being homotransplantable, and the last three being strain-specific tumors. The absorbed serums were tested against 6C3HED cells and it was found (table 3) that all these tumors completely removed the cytotoxic antibodies against 6C3HED.

TABLE 3.—In vitro *absorption of cytotoxic antibodies by cells of R2A, R2B, R4, R12, and W1**

Serum	Cells tested	Unabsorbed‡	Absorbed with†				
			R2A	R2B	R4	R12	W1
Antinormal-C3H	6C3HED	50	11	8	9	9	7
Control	6C3HED	6	6	6	6	6	6

*Antiserum produced in Swiss mice and used at a dilution of 1 in 2 with 0.08 cc. complement.
†Two absorptions for 1 hour at 37° C.
‡Percent of stained cells.

The Cytotoxic Activity of Anti-DBA and Anti-DBA-ICR

The cytotoxic activity of antiserums produced by homografts of DBA and DBA-ICR in C57BL mice was tested in a similar manner to that described for the strain C3H tumors. The results given in table 4 show that anti-DBA reacted strongly with DBA cells but showed no cytotoxic activity against DBA-ICR cells. Anti-DBA-ICR was completely devoid of any cytotoxicity, reacting neither with DBA-ICR nor with DBA cells. It seems, therefore, that the DBA-ICR cells have lost the original DBA toxinogen. This result was confirmed by absorption tests (table 5). Anti-DBA cytotoxins were completely removed by DBA cells but there was no absorption by DBA-ICR. The cytotoxic activity of anti-DBA serum was also absorbed by DBA liver cells, thus showing that the toxinogen

TABLE 4.—*Cytotoxic activity of anti-DBA and DBA-ICR**

Serum	Cells tested	Antiserum dilution 1:			Hemagglutinin titer
		Undiluted†	2^4	2^7	
Anti-DBA	DBA	87	68	15	$>2^{14}$
Control	DBA	10	11	15	
Anti-DBA	DBA-ICR	6	10	11	$>2^{14}$
Control	DBA-ICR	7	11	9	
		Antiserum 1 in 2			
Anti-DBA-ICR	DBA	6			2^8
Control	DBA	4			
Anti-DBA-ICR	DBA-ICR	8			2^8
Control	DBA-ICR	5			

*Antiserums produced in C57BL mice and used with 0.06 cc. complement.
†Percent of stained cells.

TABLE 5.—*In vitro absorption of anti-DBA antiserum by cells of DBA/2 liver, DBA, and DBA-ICR**

Serum	Cytotoxins†	Hemagglutinins
Unabsorbed	87	2^{13}
Absorbed with:		
DBA/2 liver	19	0
DBA	11	0
DBA-ICR	88	2^{13}

*Antiserum produced in C57BL mice and used at a dilution of 1 in 2 with 0.08 cc. complement. Two absorptions for 1 hour at 37° C.

†Percent of stained cells, control (normal serum) = 15.

of the DBA lymphoma is shared by normal liver cells and should therefore be regarded as part of the original antigenic pattern of DBA mice. This antigen has been lost by the DBA-ICR cells like the loss of the toxinogen of 6C3HED by 6C3HED-ICR.

The Level of Cytotoxic Antibodies in Isoimmune Serums

The titer of the cytotoxic antibodies in isoimmune serums, compared to that of the hemagglutinin titer, was determined by twofold dilutions of the antiserum (table 6). The agglutinins were detectable even above a dilution of $1:2^{14}$; the cytotoxins were no longer detectable at a much lower dilution. In the anti-DBA the agglutinin titer was $>1:2^{14}$, whereas the cytotoxin titer was $1:2^5$. The anti-MC1M also showed a similar high agglutinin titer, while the cytotoxin titer of this antiserum was $1:2^7$. As a further test on antiserums that did not produce a cytotoxic effect, anti-MC1A and anti-DBA-ICR antiserums from hyperimmune animals were tested at dilutions up to $1:2^7$. Like the dilution of 1:2, these antiserums did not produce a cytotoxic effect at any of the higher dilutions.

TABLE 6.—*Titers of the cytotoxic antibodies**

Serum	Cells tested	Antiserum dilution 1:								Hemagglutinin titer
		Undiluted†‡	2	2^2	2^3	2^4	2^5	2^6	2^7	
Anti-DBA	DBA	87	86	85	81	68	22	14	15	$>2^{14}$
Control	DBA	10	9	14	14	11	15	11	15	
Anti-MC1M	MC1M	—	67	61	64	58	46	47	29	$>2^{14}$
Control	MC1M	—	9	12	20	12	12	13	16	

*Antiserums produced in C57BL mice and used with 0.06 cc. complement.

†Percent of stained cells.

‡— = Not tested.

In Vivo *Sensitization of Tumor Cells by Cytotoxic Antibodies*

A study of the significance of the cytotoxic antibodies in the homograft reaction, *i.e.*, in the destruction of a tumor graft by the host's immune

response, was attempted by the analysis of the *in vivo* activity and function of the cytotoxic antibodies. Two opposite assumptions can be made on the possible significance of these antibodies in the isoimmune reaction. According to Gorer and O'Gorman (*3*), it is assumed, when these antibodies are active cytotoxically *in vitro* by the addition of complement, that they are also active *in vivo* without the supply of exogenous complement. The addition of complement is required due to the anticomplementary properties of mouse antiserum *in vitro*. The *in vivo* cytotoxic effect would be particularly expected, according to this assumption, with homografts of tumors such as 6C3HED and DBA, which are destroyed by the host's immune response and have been shown to produce the cytotoxic antibodies. It follows from this assumption that tumor cells taken *from a foreign strain*, when they are sensitized with the cytotoxic antibodies, should reveal the cytotoxic effect carried out *in vivo* in the foreign host merely by the application of trypan blue, and without the addition either of antiserum or of complement. On the other hand, if the antibodies do not cause a cytotoxic effect *in vivo* but receive cytotoxic properties *in vitro* by the addition of guinea-pig complement, then cells from a foreign strain, if they are sensitized with antibodies, should show cytotoxic effects after the application of complement but without treatment with antiserum. In order to test these two possibilities, the following experiment was carried out. Cells of the tumors 6C3HED, MC1M, MC1A, and 6C3HED-ICR were inoculated into hosts of their strain of origin and into hosts of foreign strains. After 9 or 11 days of growth the tumor cells were washed in Tyrode's solution and suspended in normal C3H serum similar to the suspension in antiserum in the previous experiments. Each sample of tumor cell-normal serum suspension was divided into two; one was treated with 0.08 cc. of complement and the other was examined without complement. After incubation, each of these experimental groups was treated with trypan blue.

The results (table 7) show that 6C3HED cells from C3H mice, treated with complement alone, did not show any appreciable amount of stained cells. Similar results were obtained with cells grown in Swiss mice and tested with trypan blue without the addition of complement. On the other hand, cells grown in Swiss mice but treated with complement showed 49 percent affected cells. It seems, therefore, that cells of 6C3HED grown in a foreign strain (Swiss) were sensitized with the potentially cytotoxic antibodies when drawn from the Swiss host since cytotoxic effects were revealed without the addition of antiserum. But this sensitization was not reflected *in vivo* in any cytotoxicity, because without treatment with complement no cytotoxic effects were obtained. The *in vitro* application of complement seems to have conferred cytotoxic properties on these antibodies, which by themselves do not seem to kill the tumor cells *in vivo*. A similar result was obtained with MC1M after growth in C57BL mice.

Cells of MC1A and 6C3HED-ICR did not demonstrate any cytotoxic effect when treated with complement after growth for 9 or 11 days in

TABLE 7.—In vivo *sensitization of 6C3HED, MC1M, MC1A, and 6C3HED-ICR*

Cells tested	Host strain	Number of days growth	With 0.08 cc. complement*	Without complement
6C3HED	C3H	9	5	2
	Swiss	9	49	5
MC1M	C3H	9	31	23
	Swiss	9	33	26
	C57BL	9	48	30
MC1A	C3H	9	10	3
	Swiss	9	19	10
	C57BL	9	8	4
	C3H	11	12	17
	C57BL	11	12	8
6C3HED-ICR	C3H	9	8	7
	Swiss	9	9	6
	C57BL	9	10	11

* Percent of stained cells.

hosts of a foreign strain. It was previously shown that homografts of these cells do not elicit cytotoxic antibodies. This is a further indication that cytotoxicity caused by the addition of complement is manifested only when the cells are sensitized with antibodies which they have elicited. Had these antibodies been the cytotoxic factors *in vivo*, one would expect the cells of a sensitized homograft to stain with trypan blue in contradistinction to cells of an isograft. Not having found this, one is led to the conclusion that these antibodies are not the factors responsible for the destruction of the tumor cells by the immunized mice.

Discussion

Cytotoxic Antibodies and the Homograft Reaction

The role in transplantation immunity of circulating antibodies elicited by transplants of normal and tumor tissues is still a matter of conjecture. The crucial problem in the homograft reaction, *i.e.*, in the rejection of grafted tissue by the foreign host, is the immunological mechanism by which the implanted tissue is destroyed. The isohemagglutinins produced by tissue grafts in mice and elicited by antigens determined by the same gene system that determines the histocompatibility antigens do not seem to be the antibodies that are responsible for the cytotoxic effect of the host's isoimmune response. It might therefore have been assumed that antibodies, which can be shown *in vitro* to have a lethal effect on cells that have elicited their production, would seem most likely to be involved in the homograft reaction. It is, however, well known that isoimmune serum, in general, does not seem to exert any cytotoxic effect when applied to tumor cells except in cases of leukemia (*1*, *2*).

This lack of cytotoxic effect of isoimmune serum was attributed (*3*) to the anticomplementary properties of mouse serum *in vitro*. Accord-

ingly, the addition of complement was expected to reveal the otherwise nonactive antibodies thus imitating the conditions *in vivo* in which a complement active in this system is supposed to exist. However, if these antibodies are the ones involved in the destruction of homografts in mice, one would expect passive transfer of transplantation immunity by antiserum. The transfer of antiserum will introduce the antibodies again into an *in vivo* environment containing an active complement of the normal mouse, and they should now, theoretically, be cytotoxic to cells carrying their homologous antigens. Yet it is known (*9, 10*) that such serum transfer does not confer immunity on nonimmunized mice except in cases of leukemia where the antiserum *in vitro* possesses cytotoxic properties without the addition of complement (*1, 2*). Therefore the cytotoxic antibodies demonstrated by the addition of complement *a priori* do not seem to be the main factors in the homograft reaction. This supposition was verified by the results obtained from the serological experiments in the present investigation and by results from direct tests of the intensity of transplantation immunity elicited by homografts of the tumors investigated.

Of the four ascites tumors of C3H origin, MC1A was shown (*5*) to evoke the most intense transplantation immunity measured by the increased resistance of the host to tumor homografts. A first intraperitoneal graft of MC1A into a foreign host elicited an immune response which prevented the progressive growth of a second MC1A graft, whereas a first graft of MC1M evoked a level of immunity that inhibited the growth of a subsequent MC1M but was not strong enough to resist a second MC1A. This lower immunity against MC1A cannot be attributed to the antigenic differences between the two tumors since an MC1A graft immunizes the foreign hosts against a second inoculation of MC1M. It seems, therefore, that MC1A elicits an immunity which is strong enough to resist a second MC1A, whereas MC1M does not. The transplantation immunity produced by MC1M is thus weaker than that produced by MC1A. Similar results were obtained when cross-immunity experiments were carried out with 6C3HED and MC1A. Here the transplantation immunity evoked by one graft of 6C3HED was lower than that evoked by an MC1A graft (*5*). If the cytotoxic antibodies demonstrated by the addition of complement are the factors responsible for the graft's destruction by the host's immune response, one would expect the MC1A tumor to be the most potent tumor in the production of cytotoxic antibodies. But, in fact, it was shown that this tumor neither produced these antibodies nor was it susceptible to their cytotoxic action when anti-6C3HED or anti-MC1M serums were applied to MC1A cells. Such a susceptibility would be expected if the cytotoxic antibodies are the functional antigraft factors, because 6C3HED and MC1M can immunize a foreign host to resist a second graft of MC1A (unpublished data).

A similar argument against the *in vivo* cytotoxic activity of these antibodies can be advanced for 6C3HED-ICR. It has been shown that this tumor elicits a state of transplantation immunity which is at least as in-

tense as, and probably more so than, that elicited by 6C3HED (*6*). Yet the strain-specific 6C3HED does evoke an intense host response of cytotoxic antibodies, whereas 6C3HED-ICR does not. It seems, therefore, that *the level of cytotoxic antibodies produced by a tumor homograft does not represent the level of transplantation immunity that this homograft elicits.* This discrepancy between the production of transplantation immunity by tumor homografts and their production of cytotoxic antibodies makes it doubtful that these antibodies are of importance in the homograft reaction *in vivo*.

The results of the experiments on the *in vivo* sensitization of tumor cells supports this supposition of the lack of importance of the cytotoxic antibodies in tumor-graft destruction. It has been found that cells of the 6C3HED tumor, which elicits a high level of cytotoxic antibodies when drawn out of the foreign mouse after 9 days of growth, do not show any cytotoxic symptoms. Yet these cells were shown to be sensitized with the cytotoxic antibodies in this state. Unlike cells grown in the strain of origin, the cells grown in foreign hosts revealed an increased cytotoxic effect merely by the addition of complement. Since any excess of antiserum was removed from these cells prior to the experimental treatment with complement, one must deduce that the cells themselves were sensitized with antibodies, though *in vivo* this sensitization was not reflected in any increased cytotoxicity. It is therefore suggested that these antibodies *per se* may not have any cytotoxic properties, either *in vivo* or *in vitro*, and it is only the activation by guinea-pig complement that renders them cytotoxic.

The Nature of the Cytotoxic Antibodies

The fact that 6C3HED-ICR and DBA-ICR neither elicit a production of cytotoxins nor absorb the antibodies produced by their original sublines seems to indicate that they lack the antigen(s) responsible for the production of this type of antibodies. It has previously been shown (*6*), by an analysis of the isohemagglutinins produced by these tumors, that the sublines 6C3HED-ICR and DBA-ICR lost certain antigens possessed by the original tumors. 6C3HED-ICR was shown to have lost the E agglutinogen and at least one additional agglutinogen of the *H-2* system. One could assume that the toxinogens of the tumor cells are also determined by genes belonging to the *H-2* complex in a way similar to the determination of the cell agglutinogens by these genes. Accordingly, if the loss of agglutinogens is attributed to a loss or inactivation of one or more of the *H-2* genes, this may result in a loss of other types of antigens determined by these genes, *i.e.*, the antigens responsible for the formation of the cytotoxic antibodies.

It is clear that the loss in 6C3HED-ICR of the toxinogen found in the original 6C3HED is not correlated with the loss of the E antigen. This derives from the fact that the cytotoxic antibodies elicited by homografts of 6C3HED are definitely not anti-E since the cytotoxins can be produced in C57BL mice, which possess the E antigen (*1*, *2*) and are

therefore incapable of producing anti-E antibodies. The possibility still remains that the loss of the toxinogen is correlated with the loss of the other yet undefined agglutinogen of the 6C3HED-ICR cells (*6*). Although a correlation between loss of specific agglutinogens and toxinogens has been reported in one tumor by Klein *et al.* (*11*), there is no evidence so far for such a correlation in the present study. The possibility that the toxinogens may not necessarily be directly determined by the *H-2* system is suggested by the production of cytotoxic antibodies by MC1M. It has been shown that cells of this tumor share one toxinogen with the 6C3HED cells and that they also contain one additional toxinogen which the 6C3HED cells do not possess. They are in this respect similar to normal C3H tissues. If these toxinogens are determined by the *H-2* genes, one would expect the 6C3HED cells to have lost one antigen of this system which the MC1M still possesses. The agglutinin analysis, however, does not support this conclusion. Agglutinins in anti-MC1M or anti-C3H liver are completely removed by 6C3HED cells, thus showing that MC1M or C3H liver homografts do not elicit any types of agglutinins which do not have a corresponding antigen in the 6C3HED cells (table 8).

MC1A cells neither produce cytotoxins nor are they susceptible to cytotoxins produced by 6C3HED or MC1M. However, when incubated *in vitro* with anti-MC1M, the cells of MC1A have completely removed from the antiserum the cytotoxic activity against 6C3HED and slightly decreased the activity against MC1M cells. This absorption seems immunologically specific since this high rate of antibody removal by MC1A takes place only with anti-MC1M, whereas the anti-6C3HED serum is not absorbed by MC1A cells. A tentative interpretation of this cross-reactivity between antigens of MC1A and the toxinogens of the MC1M is suggested by the assumption that MC1A cells share at least one antigen, A', with MC1M cells. This antigen is not a toxinogen since anti-MC1A serum is devoid of any cytotoxic effect. However, in order to explain the absorptive properties of MC1A cells, it is proposed that the reactive site against this A' antigen in the anti-MC1M serum is located on the same molecule that carries the cytotoxic reactive site against C', the 6C3HED toxinogen. This antibody molecule is thus

TABLE 8.—*Complete* in vitro *absorption of hemagglutinins from anti-MC1M and anti-normal-C3H liver antiserums, by cells of 6C3HED and MC1M*

Serum	Titer
Anti-MC1M	
Unabsorbed	2^{13}
Absorbed with:	
6C3HED	0
MC1M	0
Antinormal-C3H	
Unabsorbed	2^{11}
Absorbed with:	
6C3HED	0
MC1M	0

assumed to carry at least two different reactive sites, anti-A' and anti-C'.

During the incubation of anti-MC1M with MC1A cells, the A' antigen of MC1A reacts with anti-A' sites of the antibody, but the antigen-antibody complex formed on the MC1A cells will also remove the anti-C' reactive site. It is thus suggested that in the homograft reaction against the MC1M tumor, complex antibodies are formed containing on one molecule reactive sites against A' and C' antigens.

That such types of heterobivalent antibodies are feasible can be shown by the results obtained in studies of artificially conjugated antigens. Stahman *et al.* (*12*) have found that injecting rabbits with polyamino acids coupled to beef albumin resulted in the production of three distinct types of antibody molecules, one of which reacted specifically only with the polyamino acids, the second reacted with the albumin component, and the third reacted with *both antigenic determinants*. This last type of complex antibody of a heterobivalent-type molecule corresponds to our proposed anti-MC1M heterobivalent antibody. Since these complex antibodies were found only in an immune response against conjugated antigens, it seems likely that the antigens A' and C' on the MC1M cell are also of a conjugated nature, *i.e.*, located within one complex antigen molecule. In addition, MC1M has a third toxinogen, M', which would, however, be a separate molecule producing a separate antibody. A schematic presentation of this proposed pattern of the antigenic constitution of 6C3HED, MC1M, and MC1A cells is given in text-figure 1.

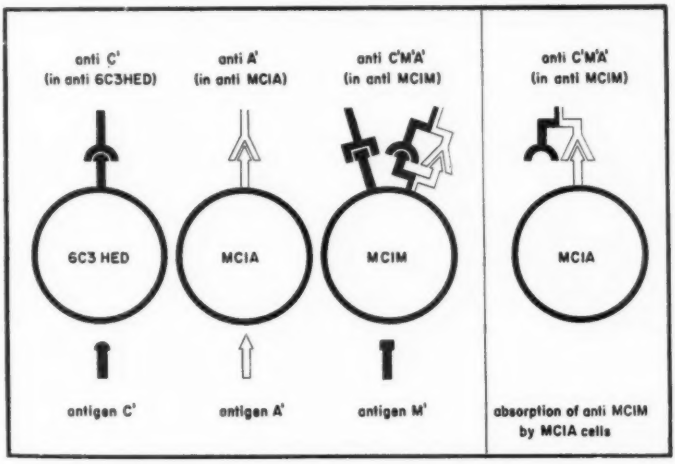
Circulating Antibodies and the Homograft Reaction

It has been well established that adoptive immunity to tissue grafts is conferred by the transfer of lymph nodes from immunized animals. The transfer of antiserum produced by tumor or normal tissue homografts, except in leukemias, does not confer a state of immunity on nonimmunized animals. The cellular transfer of transplantation immunity has led Mitchison (*9*) to the assumption that the histocompatibility antigens, which apparently do not differ to a large extent from those of the responding hosts, "may elicit antibody associated with the cells of the host, while antigens which differ more widely may elicit serum antibody." This lack of transfer of immunity by antiserum was, in fact, the main basis for the analogy between the homograft reaction and the delayed type of hypersensitivity (the tuberculin type of allergy). A state of delayed sensitivity cannot be transferred by antiserum, though it can be transmitted by cells. Furthermore, other similarities between the two types of immune reaction can be pointed out (*13*): 1) The tuberculin type of allergic response, like tissue transplantation immunity, is elicited by the intradermal rather than by the intravenous inoculation of the antigen. 2) The antigenic stimulus in both types of immune reactions is carried normally by cells. 3) The latent period of the host's response in delayed sensitivity (10 to 14 days) is similar to the latent period obtained by skin homografts.

However, these similarities do not exclude the possible role of serum antibodies in the homograft reaction. It has been found (*14*) that agammaglobulinemic patients do not reject a skin graft, though they can develop delayed allergy to tuberculin (*15*). Delayed hypersensitivity can thus be formed under conditions in which no circulating antibodies are formed, while a homograft destruction is obtained only in animals that do produce serum antibodies. One may assume that in agammaglobulinemia the mechanism responsible for globulin deficiency is also responsible for the incapability of the "cellular immune response." But if this were the case, then the tuberculin hypersensitivity, depending also on cellular response, would be similarly impaired in agammaglobulinemia. It therefore seems probable that circulating antibodies participate in the homograft reaction to a greater extent than in the tuberculin allergic response.

It has been shown that the transfer of immunized lymph-node cells produces circulating antibodies detected as isohemagglutinins (*16*). It is possible that other types of antibodies are formed by the implanted lymphoid cells, and that some of these may be involved in the destruction of the grafted tissue. Gorer has postulated (*17*) that antibodies may act synergistically with leukocytes. Billingham (*18*) has also found some effect of isoimmune serum on skin cells. This evidence, therefore, makes the study of circulating antibodies produced in the isoimmune response a relevant subject in the analysis of the mechanism of the homograft reaction.

It has been shown in the present study that the cytotoxic antibodies activated by complement are not the serum antibody component involved in the graft destruction. It seems probable that the dominant "cyto-



TEXT-FIGURE 1.—Diagrammatic representation of the distribution of the *C'*, *M'*, and *A'* antigens in cells of the 6C3HED, MC1M, and MC1A tumors. The *C'* and *M'* antigens are toxinogens, whereas the *A'* antigen is not toxinogenic.

toxic" factors in the host's immune response are the lymphoid cells or perhaps "intracellular antibodies" in these cells (*19*). But there is also the possibility that serum antibodies may function as cofactors in the homograft reaction, and that they, themselves, therefore need not possess cytotoxic properties.

References

(*1*) Gorer, P. A.: Some recent work on tumor immunity. Advances Cancer Res. 4: 149–186, 1956.

(*2*) Amos, D. B., and Day, E. D.: Passive immunity against four mouse leukoses by means of isoimmune sera. Ann. New York Acad. Sc. 64: 851–858, 1957.

(*3*) Gorer, P. A., and O'Gorman, P.: The cytotoxic activity of isoantibodies in mice. Transpl. Bull. 3: 142–143, 1956.

(*4*) Sachs, L., and Gallily, R.: The chromosomes and transplantability of tumors. III. The transplantability of mouse tumors with different degrees of strain specificity into previously immunized mice. J. Nat. Cancer Inst. 16: 1083–1097, 1956.

(*5*) Feldman, M., and Sachs, L.: The antibody response to successful tumor homografts. J. Nat. Cancer Inst. 18: 529–553, 1957.

(*6*) ———: Immunogenetic properties of tumors that have acquired homotransplantability. J. Nat. Cancer Inst. 20: 513–539,1958.

(*7*) Sachs, L., and Gallily, R.: The chromosomes and transplantability of tumors. II. Chromosome duplication and the loss of strain specificity in solid tumors. J. Nat. Cancer Inst. 16: 803–841, 1956.

(*8*) Gorer, P. A., and Mikulska, Z. B.: The antibody response to tumor inoculation. Improved methods of antibody detection. Cancer Res. 14: 651–655, 1954.

(*9*) Mitchison, N. A.: Passive transfer of transplantation immunity. Proc. Roy. Soc., London. ser. B 142: 72–87, 1954.

(*10*) Billingham, R. E., Brent, L., and Medawar, P. B.: Quantitative studies on tissue transplantation immunity. II. The origin, strength and duration of actively and adoptively acquired immunity. Proc. Roy. Soc., London. ser. B 143: 58–80, 1954.

(*11*) Klein, E., Klein, G., and Révész, L.: Permanent modification (mutation?) of a histocompatibility gene in a heterozygous tumor. J. Nat. Cancer Inst. 19: 95–114, 1957.

(*12*) Stahmann, M. A., Tsuyuki, H., Weinke, K., Lapresle, C., and Grabar, P.: L'antigenicité des polypeptides synthetiques. Compt. rend. Acad. sc., 241: 1528–1529, 1955.

(*13*) Lawrence H. S.: Similarities between homograft rejection and tuberculin-type allergy: A review of recent experimental findings. Ann. New York Acad. Sc. 64: 826–835, 1957.

(*14*) Good, R. A., Varco, R. L., Aust, J. B. and Zak, S. J.: Transplantation studies in patients with agammaglobulinemia. Ann. New York Acad. Sc. 64: 882–924, 1957.

(*15*) Porter, H. M.: The demonstration of delayed-type reactivity in congenital agammaglobulinemia. Ann. New York Acad. Sc. 64: 932–935, 1957.

(*16*) Mitchison, N. A., and Dube, O. L.: Studies on the immunological response to foreign tumor transplants in the mouse. II. The relation between hemagglutinating antibody and graft resistance in the normal mouse and mice pretreated with tissue preparations. J. Exper. Med. 102: 179–197, 1955.

(*17*) Gorer, P. A.: The value of ascites tumors in problems of tumor immunity. Ann. New York Acad. Sc. 63: 882–892, 1956.

(*18*) Billingham, R. E.: Studies on epidermal cell suspensions, with particular reference to problems of transplantation immunity. Ann. New York Acad. Sc. 64: 799–806, 1957.

(*19*) Voisin, G. A., and Maurer, P.: Studies on the role of antibodies in the failure of homografts. Ann. New York Acad. Sc. 64: 1053–1070, 1957.

The Effect of Inoculum Size on Proliferation of NCTC Strain 2071, the Chemically Defined Medium Strain of NCTC Clone 929 (Strain L)[1]

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Summary

Replicate culture and nuclei enumeration procedures were used to study the effect of inoculum sizes of NCTC strain 2071, the chemically defined medium strain of NCTC clone 929 (strain L). The cultures were grown in T-15 flasks containing 3.0 ml. of NCTC 109 medium which contains no added protein. The replicate culture procedure was altered slightly to compensate for the omission of serum; such alterations are described. Initial cell density ranged from 15,500 to 2,001,900 per 3.0 ml. Inoculums between 15,500 and 36,600 were unpredictable with respect to survival and proliferation, but even with an inoculum of 15,500 some cultures proliferated. Inoculums of 36,600 or more per 3.0 ml. showed proliferation of all cultures in each set. Between 36,600 and 1,031,200, the rate of proliferation was never less than 2 at 7 days. The optimum range of inoculum size appeared to be between 160,000 and 410,000. The rate of proliferation of cultures inoculated with optimum densities was highest of all. The maximum cell population attained per culture was not dependent on the initial cell inoculum. **J. Nat. Cancer Inst. 21: 579–583, 1958.**

Physiological studies involving cell assay would be valueless if growth failures were due to inoculums of inadequate size. Previous studies (*1*, *2*) demonstrated not only the maximum and minimum sizes of inoculums required by 2 different strains of mammalian cells *in vitro* but also the influence of the inoculum size on proliferation rates. Recording this information on strain NCTC 2071 has become particularly pertinent as that line of strain L has been maintained for 3 years in a chemically defined medium free of any added protein and has therefore become increasingly useful in a variety of physiological investigations.

The present study determined the following for NCTC strain 2071: 1) the size of inoculum that will yield any proliferation, 2) the size that will

[1] Received for publication April 1, 1958.

[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

468155—58——14

uniformly and consistently give 100 percent proliferation, and 3) the rate of population increase.

Materials and Methods

Replicate cultures of NCTC strain 2071 (*3*, *4*) were prepared, and nuclei enumerated, as previously described (*5*, *6*). The replicate culture procedure was altered slightly by the use of an 0.1 percent methylcellulose-saline solution (*7*) as the diluent during the collection and centrifugation of cells and as the suspending fluid. The cells were centrifuged for 20 minutes at 500 r.p.m. (approximately 45 × g) and resuspended in fresh methylcellulose-saline solution, after which they were stirred at 450 r.p.m. for a minimum of 20 minutes prior to planting. Aliquots of cell suspension 0.5 (±0.02) ml. were then delivered into T-15 flasks (*8*) containing 2.5 ml. NCTC 109 (*4*, *9*) planting medium,[3] and therefore grown in the complete NCTC 109 on the glass floor of the flask. Immediately before fluid renewals, the flasks were centrifuged at 500 r.p.m. for 20 minutes, and then 2.0 ml. of exhausted medium was quantitatively removed and replaced with 2.0 ml. of fresh medium. Cultures planted from each suspension were considered an experimental set.

Nuclei were enumerated on inoculums and on cultures maintained for 7 days and, in several instances, 17 hours and 14 days *in vitro*. Such enumeration was accomplished within 2 weeks[4] after fixation of the cells with citric acid. The rate of proliferation[5] and the percent increase[6] in population were calculated.

Results

Results collected on cultures cultivated for 1 week are recorded in table 1. In addition to the population levels of inoculums, the table gives the range and average final populations at 7 days, the percent of cultures proliferating, the rate of proliferation, and the actual percent increase.

Some proliferation was obtained from each inoculum studied. With only 3 exceptions, 100 percent of the cultures proliferated. Until the inoculum population was in excess of 1,000,000, the final population increase was uniformly more than 100 percent and the rate of proliferation was never less than 2 at 7 days.

Above an inoculum population of 550,000, the final maximum population was usually greater than that obtained with the lesser sizes of inoculums; the maximum cell population therefore appeared dependent on the original inoculum under these experimental conditions. The optimum

[3] The planting medium was NCTC 109 whose components were concentrated appropriately so that upon the addition of the 0.5 ml. methylcellulose-saline-cell suspension, the medium was complete NCTC 109 of the proper concentration.

[4] Statistical evaluation to validate the 2-week enumeration as compared with the 1-week counts revealed no statistical variation between them.

[5] Rate of proliferation per time interval was determined according to the method used by Earle *et al.* (*1*) for the parent strain NCTC clone 929 by dividing the average final population by the average inoculum.

[6] Percent increase of cultures was calculated by dividing the difference between the average final population and the average original inoculum by the average inoculum, and then multiplying by 100.

TABLE 1.—*Relationship of inoculum size of cultures of NCTC 2071 cells to population at 7 days*

Inoculum population (*nuclei per flask*)	Number of cultures			Final population in thousands		Percent increase	Rate of proliferation
	Counted	Proliferating	Percent proliferating	Range	Average		
15,500	5	2	40	11–24	16	—	—
32,300	5	3	60	13–44	30	—	—
32,700	5	1	20	16–33	24	—	—
36,600	5	5	100	58–122	87	136	2.1
84,900	5	5	100	116–357	215	153	2.5
111,700	5	5	100	215–513	330	195	2.9
167,500	5	5	100	1084–1370	1220	628	7.3
170,200	4	4	100	1874–2520	2234	1213	13.1
247,300	5	5	100	1640–1887	1808	631	7.3
341,400	5	5	100	1519–2391	1770	419	5.2
364,600	10	10	100	669–1160	902	147	2.5
364,700	4	4	100	3119–3528	3398	832	9.3
407,200	4	4	100	3554–3987	3782	829	9.3
459,900	4	4	100	907–1739	1313	186	2.9
504,900	8	8	100	1300–2280	1626	222	3.2
572,000	8	8	100	729–1622	1176	106	2.1
582,200	6	6	100	1066–1818	1385	138	2.4
602,000	4	4	100	1673–2853	2234	271	3.8
700,300	4	4	100	2138–3464	2657	280	3.8
715,600	3	3	100	2027–2733	2288	220	3.2
888,800	6	6	100	1460–2707	2322	161	2.6
928,300	6	6	100	2720–3456	3050	229	3.3
972,000	6	6	100	2544–4278	3328	242	3.4
1,031,200	4	4	100	3093–3874	3488	238	3.4
1,329,900	3	3	100	2047–2633	2422	82	1.8
2,001,900	6	6	100	2620–4176	3227	61	1.6
Total	135	126					

range of inoculums appeared to be between 160,000 and 410,000. Here the rate of proliferation usually ranged between 5 and 13 times the inoculum at 7 days.

Some proliferation was observed in most cultures from the several inoculums on which 17-hour growth data were collected. The average final proliferation was greater than the inoculum population in each set. The calculated rate of increase was less than that calculated for cultures planted from the same inoculum suspension but maintained for 7 days. For example, with an inoculum of 364,734, one hundred percent of the cultures proliferated and the rate of increase was 1.366 in contrast to 9.287 at 7 days.

In contrast to the 7-day data on percent of increase in population and rate of proliferation, those from 14-day cultivation showed some differences. When cultures were maintained for 2 weeks, even an inoculum greater than 1 million but less than 2 million gave at least 100 percent increase in population and a rate of proliferation not less than 2 at 14 days. With inoculums of less than 1 million, however, the percent increase in population and the rate of proliferation for 2-week cultures was similar to that obtained in 1-week cultures.

Discussion

With NCTC strain 2071 maintained for 7 days in 3.0 ml. of NCTC 109 medium in T-15 flasks (*8*), proliferation of every culture in every set was obtained, with only 3 exceptions. Under these conditions, the minimum population of 36,600 of NCTC 2071 cells necessary for reproducible culture proliferation was less than the 100,000 reported by Earle *et al.* (*1*) as being required for the parent strain NCTC clone 929 cells, in 2.0 to 2.5 ml. of a stock medium of dilute horse serum and embryo extract in T-12 flasks. Pace and Aftonomos (*2*) found that the minimum inoculum size with NCTC clone 1469 of liver parenchymal cells was 105,000 for 100 percent proliferation of all cultures. Their study was carried out in D-3.5 Carrel flasks and the cells were cultured in 1.0 ml. of a dilute horse serum and embryo-extract medium.

The behavior of inoculums exceeding 2 million was not investigated in the present study but, at the 2 million nuclei inoculum level, proliferation of 100 percent of the cultures was obtained. The data of Earle *et al.* on the maximum size of inoculum of NCTC clone 929 which would give 100 percent proliferation of the cultures in a set were in this range. With the liver cells, inoculum populations above 1,425,000 were unpredictable in survival and proliferation.

The data on the percent of increase and the rate of proliferation from the present study disclosed certain facts. Inoculum populations between 36,000 and 1 million gave a rate of increase ranging from 2- to 13-fold the inoculum. Proliferation in terms of percent-population increase was never less than 100 percent and generally 200 percent or greater.

At inoculum levels less than 36,000 and more than 1 million the rate of proliferation was less than 2 at 7 days. Through a wide range of inoculums the final populations attained approximately similar maxima. The optimum range of inoculums appeared to be between 160,000 and 410,000; in this range the rate of population increase was the greatest.

Determination of inoculum sizes for NCTC 2071 that will uniformly and consistently yield 100 percent proliferation obviates the necessity of employing the statistical device of determining outliers resulting from inadequate inoculum population. This information is of prime importance when this NCTC 2071 line of clone 929 (strain L) is used for physiological studies.

It is clearly recognized that the NCTC 929 and NCTC 1469 cells are different from the NCTC 2071 cells, and that the actual cultural conditions are different, and, therefore, that factors other than volume of fluid may be involved in the size of inoculum population that will proliferate 100 percent. In summary, however, it may be noted that, while 50,000 NCTC 929 cells per ml. and 105,000 NCTC 1469 cells per ml. are required for 100 percent proliferation, only 12,000 NCTC 2071 cells are required per ml. of NCTC 109 for 100 percent proliferation.

References

(1) EARLE, W. R., SANFORD, K. K., EVANS, V. J., WALTZ, H. K., and SHANNON, J. E., JR.: The influence of inoculum size on proliferation in tissue cultures. J. Nat. Cancer Inst. 12: 133–153, 1951.

(2) PACE, D. M., and AFTONOMOS, L.: Effects of cell density on cell growth in a clone of mouse liver cells. J. Nat. Cancer Inst. 19: 1065–1075, 1957.

(3) EVANS, V. J., BRYANT, J. C., MCQUILKIN, W. T., FIORAMONTI, M. C., SANFORD, K. K., WESTFALL, B. B., and EARLE, W. R.: Studies of nutrient media for tissue cells *in vitro*. II. An improved protein-free chemically defined medium for long-term cultivation of strain L-929 cells. Cancer Res. 16: 87–94, 1956.

(4) MCQUILKIN, W. T., EVANS, V. J., and EARLE, W. R.: The adaptation of additional lines of NCTC clone 929 (strain L) cells to chemically defined protein-free medium NCTC 109. J. Nat. Cancer Inst. 19: 885–907, 1957.

(5) EVANS, V. J., EARLE, W. R., SANFORD, K. K., SHANNON, J. E., JR., and WALTZ, H. K.: The preparation and handling of replicate tissue cultures for quantitative studies. J. Nat. Cancer Inst. 11: 907–927, 1951.

(6) SANFORD, K. K., EARLE, W. R., EVANS, V. J., WALTZ, H. K., and SHANNON, J. E., JR.: The measurement of proliferation of tissue cultures by enumeration of cell nuclei. J. Nat. Cancer Inst. 11: 773–795, 1951.

(7) FIORAMONTI, M. C., BRYANT, J. C., MCQUILKIN, W. T., EVANS, V. J., SANFORD, K. K., and EARLE, W. R.: The effect of horse serum residue and chemically defined supplements on proliferation of strain L clone 929 cells from the mouse. Cancer Res. 15: 763–766, 1955.

(8) EARLE, W. R., and HIGHOUSE, F.: Culture flasks for use with plane surface substrate tissue cultures. J. Nat. Cancer Inst. 14: 841–851, 1954.

(9) EVANS, V. J., BRYANT, J. C., FIORAMONTI, M. C., MCQUILKIN, W. T., SANFORD, K. K., and EARLE, W. R.: Studies of nutrient media for tissue cells *in vitro*. I. A protein-free chemically defined medium for cultivation of strain L cells. Cancer Res. 16: 77–86, 1956.

Effect of a Bacterial Polysaccharide and of Several Other Tumor-Necrotizing Agents on Carotid Blood Pressure in Mice [1, 2]

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Summary

The effect of several tumor-necrotizing agents on systemic blood pressure in mice was studied by a method that permitted continuous recording of pressure directly from the carotid artery. The polysaccharide from *Serratia marcescens* did not depress the blood pressure immediately. When the continuous recording was prolonged for hours, a gradual fall of blood pressure was observed in the control as well as in the treated mice. However, when the recording was limited to a half-hour period a significant fall was not produced within the first 6 hours after administration of polysaccharide. Analogously, the tracings obtained in mice treated with podophyllotoxin, colchicine, and several of their derivatives showed no evidence of induced fall of blood pressure on comparison with the tracings yielded by the controls. The production by these drugs of a sustained systemic hypotension, which had been postulated as a necessary prerequisite for their elicitation of hemorrhagic necrosis in tumors, is not supported by this study. **J. Nat. Cancer Inst. 21: 585-594, 1958.**

On the basis of the measurement of peripheral blood pressure, with an indirect method, in small vessels of the skin near implanted tumors in the mouse, Algire and coworkers (*3–6*) postulated that prolonged hypotension was the primary effect prerequisite for the induction of hemorrhagic necrosis in tumors following injection of bacterial polysaccharide and certain other agents. Measurement of systemic blood pressure directly from a major artery should provide a more pertinent basis for evaluation of the role of hypotension in the induction of this tumor reaction. Accordingly, we developed a method (*7*) for the continuous recording of the systemic blood pressure from the carotid artery of mice. Inasmuch as such information had hitherto not been available for mice,

[1] Received for publication April 8, 1958.

[2] Preliminary reports (*1, 2*) of this work were given at the fall, 1954, and spring, 1955, meetings of the American Society for Pharmacology and Experimental Therapeutics.

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[4] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

some of the characteristics of carotid blood pressure in this species were studied (*7*), as well as the response to drugs of different pharmacologic nature (*8*).

This paper presents data on the effect of a bacterial polysaccharide, podophyllotoxin, colchicine, and some of their derivatives on carotid blood pressure in both normal and tumor-bearing mice. Observations of this kind likewise have not hitherto been obtainable in mice, and the present results represent the first efforts in this direction. The initial levels of the blood pressure were obtained under experimental conditions consisting of a large dose of urethan (for the prolonged anesthesia) and of the surgical procedures necessary for exposure of the carotid artery. When the continuous recording was prolonged for hours, there was a gradual decrease in the blood pressure, and the mortality rose, both in the controls and in the treated mice. However, when each recording period was limited to one-half hour, the data obtained during the first 6 hours following administration of the polysaccharide did not provide evidence of a severe and prolonged primary hypotension.

Materials and Methods

CA F_1 mice of both sexes weighing 20 to 28 gm. were used. Carotid blood pressure was recorded and measured as described (*7*) previously. The carotid artery was dissected under urethan or, occasionally, sodium pentobarbital anesthesia and a 27-gauge needle inserted. The needle was fitted directly to a Statham physiological pressure transducer (model P-23D), which was attached to a Brush Universal Analyser; this, in turn, was connected to a Brush Oscillograph on which pressure was recorded by a direct writing pen. The level of blood pressure was read in mm. of Hg from the calibration curve made at the beginning of each experiment. Most of the measurements were made in mice which bore 6-day-old implants of Sarcoma 37 subcutaneously or intramuscularly.

When the blood pressure became stabilized (usually within one-half hour) a tumor-necrotizing agent, or an inactive control material, was injected intraperitoneally, intravenously, or intra-arterially; its effect on blood pressure was observed until, in most cases, the death of the mouse. The agents included: polysaccharides from *Serratia marcescens* (lot #P25, *9*) and from a variety of animal and plant sources (mouse liver, mouse Lymphoid leukemia L1210, shrimp, tobacco, and cantaloupe; *10–12*); podophyllotoxin (*13*); acetylpodophyllotoxin-ω-pyridinium chloride (NCI #3022, *14*); and cochicine and 3 of its derivatives (*15–17*). The dosage employed ranged upward from the minimum amount effective in inducing tumor damage (*18*).

Results

Control Mice

As a basis for comparison with the effects produced by the agents administered, the blood pressure was continuously recorded in 53 un-

treated mice, 19 of which bore tumor implants. The depressant effect of the anesthetic and of the surgery was shown by the increasing mortality, accompanied by a gradual fall of blood pressure in most of the mice, as the recording continued. Table 1 gives a summary of the findings during the first 3 hours. Seven of the 53 mice were still alive after 6 hours of continuous recording.

TABLE 1.—*Blood-pressure changes in control mice and survival during prolonged continuous recording*

Mice	Recording period (*hours*)	Number of mice			
		Alive at start of period	In which blood pressure:*		
			Rose	Remained unchanged	Fell
Without tumor	0 –0. 5	34	10	3	21
	0. 5–1	34	3	0	31
	1 –2	26	3	1	22
	2 –3	14	1	1	12
Tumor-bearing	0 –0. 5	19	8	1	10
	0. 5–1	19	5	1	13
	1 –2	17	2	2	13
	2 –3	11	1	0	10

*For detailed findings in such mice *see* (7).

Polysaccharides

A single injection of the *S. marcescens* polysaccharide was given to 65 mice, of which 42 bore implants of Sarcoma 37; the dose ranged between 0.5 and 10 μg. per gm. of body weight. For the most part, the polysaccharide was injected intraperitoneally but the intravenous and intra-arterial routes were also employed for comparison. Since the results were similar they are summarized together (table 2).

TABLE 2.—*Blood-pressure changes in mice and survival during prolonged continuous recording after injection of bacterial polysaccharide**

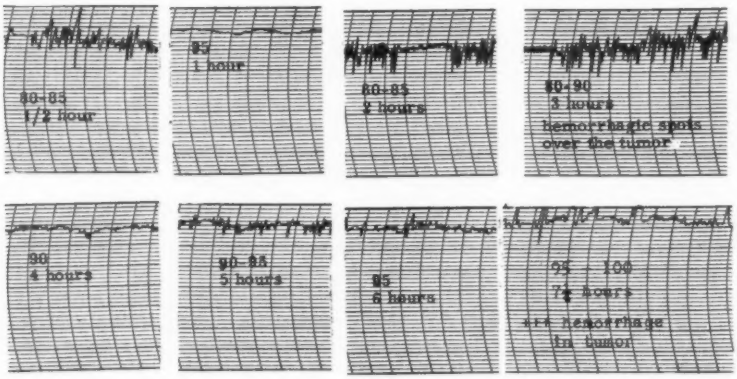
Mice	Recording period (*hours*)	Number of mice			
		Alive at start of period	In which blood pressure:*		
			Rose	Remained unchanged	Fell
Without tumor	0 –0. 5	23	11	7	5
	0. 5–1	22	10	4	8
	1 –2	16	2	5	9
	2 –3	7	3	2	2
Tumor-bearing	0 –0. 5	42	21	11	10
	0. 5–1	34	7	9	18
	1 –2	30	4	4	22
	2 –3	20	3	3	14

*Dose was varied between 0.5 and 10 μg. per gm. of body weight.

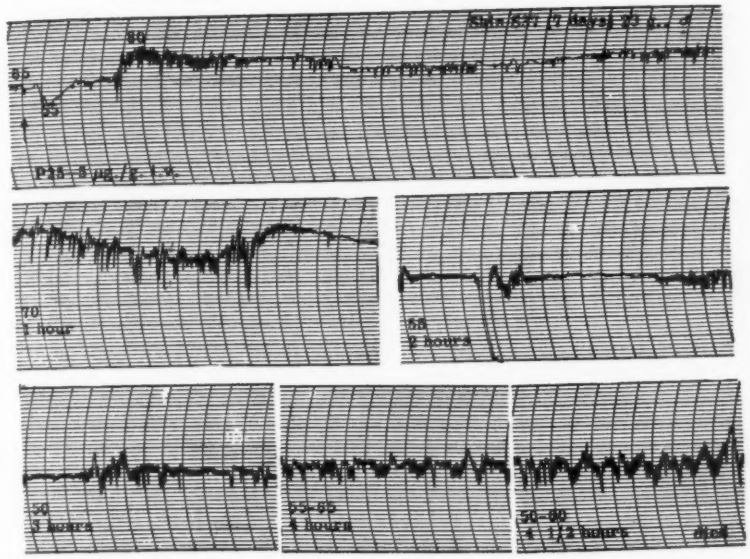
On comparison with the controls (table 1) it is seen that the polysaccharide did not depress the blood pressure during the first few hours; in fact, a larger proportion of the mice showed no change in blood pressure. Moreover, a greater proportion of the mice showed a rise following the polysaccharide injection than was the case in the control animals.

After 3 hours of continuous recording, the mortality in the treated mice continued, as in the controls, but at the end of 6 hours 5 of the mice were still alive. The tracings obtained for one tumor-bearing mouse that was sacrificed after 7¼ hours are shown in text-figure 1. It is obvious that no drop in blood pressure, either sudden or gradual, was produced by the polysaccharide (1 μg./gm., intraperitoneal) between one-half hour and the time the experiment was terminated by sacrificing the animal. At this time the usual hemorrhage, induced in the tumor after this interval following injection of polysaccharide, was strikingly evident through the skin. The tracings for another mouse, which died 4½ hours after an intravenous injection of a fivefold greater dose, are shown in text-figure 2. Even at this quite high dose (*i.e.*, 115 μg./mouse) no hypotension developed until 2 hours had elapsed; immediately after the injection the momentary fall in blood pressure was followed by a rise which persisted for about 1 hour.

In these experiments, in which recording was carried out continuously for a long time, the mortality was so high that only a few animals survived for 6 hours—the period required for the induced hemorrhage to become evident. Hence these data could not serve for correlating tumor damage with any other parameter. Therefore another approach was designed. The dose of polysaccharide employed, in the work next to be described, was 1 μg. per gm. of body weight given intraperitoneally. This dose was well above the amount that regularly induced severe hemorrhage and necrosis in the tumors of the thousands of mice which had received this preparation (lot #P-25) over many years.



TEXT-FIGURE 1.—Blood-pressure recording in a 25 gm. mouse bearing a 6-day-old intradermal implant of Sarcoma 37. Recording started one-half hour after intraperitoneal injection of 1 μg. per gm. of *Serratia marcescens* polysaccharide.



TEXT-FIGURE 2.—Blood-pressure recording in a 23 gm. mouse bearing a 7-day-old intradermal implant of Sarcoma 37. Recording started shortly before intraperitoneal injection of a high dose (5 μg./gm.) of *Serratia marcescens* polysaccharide.

In the foregoing experiments, the polysaccharide was administered after the surgery was performed in the anesthetized animals. Consequently, the recordings obtained several hours after treatment with polysaccharide were values for animals that had been subjected to severe, long-continued stresses. To minimize the deleterious effects of such stresses, an alternative procedure was employed in 65 tumor-bearing mice.

In these animals, the polysaccharide was given first. At various intervals after injection, the surgery was performed and the pressure recorded until it was stabilized, *i.e.*, for only about one-half hour. Table 3 summarizes the data obtained in this way, which limited the period of stress to a maximum of 1 hour. Comparison with the untreated controls shows that during the first 6 hours following administration the polysaccharide did not produce a significant hypotension. This is the period during which induced hemorrhagic necrosis becomes evident in the tumors.

The effect of the bacterial polysaccharide on blood pressure was explored in a small number of rats, both normal and tumor-bearing. An osteogenic sarcoma, found to be responsive to this agent, was the tumor employed. The pressure was recorded directly from the carotid artery of normal and tumor-bearing rats anesthetized with urethan and, with a different method (*19*), from the abdominal aorta [5] of unanesthetized tumor-bearing rats. In the anesthetized rats the pressure was recorded continuously until death; in the others it was recorded for short periods during

[5] The authors are indebted to Mr. Ernest R. Whitcomb for preparing some of the rats used.

TABLE 3.—*Mean blood pressure in mice* recorded for only one-half hour at various intervals after polysaccharide administration*

Time in hours after injection of polysaccharide:†							
0 (*untreated*)	1	2	3	4	5	6	24
(*mm.*)	(*mm.*)	(*mm.*)	(*mm.*)	(*mm.*)	(*mm.*)	(*mm.*)	(*mm.*)
70	60	110	75	55	90	95	90
90	100	65	105	90	75	95	100
60	100	100	55	60	90	75	100
110	100	90	80	80	80	100	80
85	90	110	85	90	75	45	65
85	85	80	110	65	62	80	90
78	90	92	75	85	80	55	90
80		100	60	80			85
80			75	50			90
82 ±5	89 ±5	93 ±5	80 ±6	73 ±5	79 ±3	78 ±8	88 ±3

*A separate mouse bearing a 6-day-old implant of S37 was used for each of the 65 determinations.
†*S. marcescens* polysaccharide; 1 μg. per gm. intraperitoneal.

the first 4 or 5 hours and again after 24 hours. The tracings showed either no change or only a slight fall in blood pressure.

The effects of 5 other polysaccharides mentioned earlier were examined in 2 or 3 mice each. These preparations were selected on the basis of their tumor-damaging potency that ranged from none, through weak, to strong. During the first 2 hours the blood pressure in most of the mice showed no change from the initial level or even a slight rise. No evidence of correlation was seen between potency and blood-pressure effect.

Podophyllotoxin and a Derivative

Podophyllotoxin in a dose of 5 to 10 μg. per gm. was injected subcutaneously, intraperitoneally, or intravenously in mice. For a prolonged period after injection the blood pressure in most of the animals either rose slightly or did not change.

Acetylpodophyllotoxin-ω-pyridinium chloride in a dose of 10 to 20 μg. per gm. was injected intraperitoneally or intravenously. Although this dose level was not tolerated by some of the mice under these conditions, the pressure in most of them showed no change or a slight rise during the 1st hour; only in an occasional mouse did it drop for a few minutes.

Colchicine and its Derivatives

Colchicine and 3 of its derivatives—trimethylcolchicinic acid (TMCA) methyl ether *d*-tartrate (NCI #1136), colchiceinamide (NCI #2177) and demecolcine (NCI #3371)—were also examined. All were effective (*15–17*) in causing hemorrhagic necrosis in Sarcoma 37.

Colchicine (100 μg./gm., intraperitoneally) did not produce a significant change in blood pressure. TMCA methyl ether *d*-tartrate (10 to

100 μg. per gm., intraperitoneally or intravenously) caused a rise of blood pressure, to a variable extent, until shortly before death (between 10 minutes and 2 hours). Colchiceinamide (10 to 80 μg. per gm., intraperitoneally or intravenously) caused either no change or a slight rise of pressure in a majority of the cases. A fall was observed only in a few mice. Demecolcine (50 to 110 μg. per gm., intravenously) caused a fall of 10 to 25 percent, which persisted for 15 minutes to an hour or more in some of the mice. In one case the maximum fall was about 40 percent; in a few others there was either no change or even a slight rise.

Discussion

The carotid blood pressure in mice, when measured with the method employed here, usually responded (*8*) to known pressor and depressor drugs as in cats and dogs. Moreover, the response was prompt, again as in the larger animals.

In the present investigation the response of blood pressure in mice to several tumor-necrotizing agents was studied. The tracing patterns yielded by mice, with or without tumors, after injection of these agents were compared with those obtained in the controls; production of an immediate drop in blood pressure by these agents was not evident. Hence, no support was obtained for the alleged primary hypotensive effect (*3–6*).

In cancer patients (*20–21*) the blood pressure frequently declined after an injection of the bacterial polysaccharide. However, this decline did not start immediately; usually the blood pressure rose at first and then began to fall only after some time had elapsed. This is interpreted as indicating that the polysaccharide is not a depressor drug, but that the fall in blood pressure is a secondary reaction which develops in consequence of some antecedent primary effect.

In the early 1940's one of us (MJS) collaborated with Dr. Stanley P. Reimann and others in a clinical study[6] at the Lankenau Hospital in Philadelphia (*21*). In the cancer patients who received this bacterial polysaccharide intravenously, the temperature usually rose to a peak in 4 to 5 hours while the blood pressure usually fell, after an initial rise, with the low point also at about 4 to 5 hours. However, in one patient neither the temperature nor the blood pressure changed much, and yet this was the patient in whom there occurred the most pronounced recession of the multiple tumors. This result, also, cannot be reconciled with the hypotention theory.

In the mice that had been given polysaccharide the absence of an immediate drop in blood pressure also indicated the absence of a primary hypotensive action. The later effects on blood pressure were no different from those in the controls. Nevertheless, the later period presents only a tentative picture until such time as a method is devised that permits maintenance of the blood-pressure level in control mice during prolonged recording.

[6] The details of the study of these and subsequent patients will be published separately by the Philadelphia group.

The foregoing considerations apply to the situation in which the pressure is continuously recorded for many hours. An attempt was made to avoid the drop in pressure, seen even in the controls (table 1), consequent to the stress of the test conditions in prolonged recording. This was accomplished by recording the pressure for only one-half hour, in separate mice, at various intervals after injection of the polysaccharide; as is shown in table 3 the progressive drop in blood pressure during continuous recording, in both controls and treated mice, was not encountered. Thus, delaying the anesthesia and the surgery until immediately prior to measurement of the carotid blood pressure and recording it for only a short period minimized the various deleterious effects. Under these more favorable conditions it was found that the blood pressure did not sustain a significant fall during the 6 hours following injection of polysaccharide. In fact, during the first 2 hours there was an *increase* in the mean values; it was only later that they were lower than the initial ones. However, neither the rise nor the fall was markedly significant when considered in the light of the standard errors. Such observations do not confirm the correlation that has been postulated between the action of this material on systemic blood pressure and its tumor-damaging property.

Some of our previous findings (*22*) indicated that vasoconstriction appeared to play a role in the elicitation of hemorrhagic necrosis in tumors by polysaccharides and some other agents, but all the observations (*8*) could not be interpreted on this basis. It would appear that vasoconstriction and/or other circulatory disturbances evoked *in the region of the tumor* may be involved in the induction of this phenomenon, but the agents which produce such localized vascular effects need not necessarily affect systemic blood pressure.

Among the local changes observed by Algire and coworkers in and around tumor implants in a skin flap were: slowing of blood flow, followed by stasis; edema in the subcutaneous tissue and in the tumor; and reduction of the pressure (as measured by an indirect method) in small blood vessels near the tumor. These changes, observed in a transparent chamber following administration of the tumor-necrotizing agents, were succeeded by hemorrhage and necrosis in the tumors. The entire sequence of events was ascribed to "circulatory slowing throughout the host" as the primary effect of the bacterial polysaccharide; it was postulated that ". . . the mechanism of action of this agent is an example of a host reaction to a variety of agents which similarly decrease the host circulatory rate, secondarily affecting the tumor circulation . . . ."

The uncertainties surrounding blood-pressure data obtained with indirect methods are resolved only when the latter are validated by comparisons with direct measurements. It is not known to what extent a slowing of the rate of blood flow and a drop in pressure in small vessels of the skin may be employed as reliable measures of circulatory slowing and reduced pressure throughout the host.

Drugs like dibenamine and dibenzyline lower the blood pressure in mice (*8*) as they do in larger animals. If the mechanism by which the bacterial

polysaccharide damages tumors required it to produce a prolonged antecedent hypotension, one might expect that dibenamine and dibenzyline would enhance the tumor damage. Yet we have found that each of these drugs *blocks* the antitumor action of the polysaccharide (*22*).

Some anticancer agents are directly cytotoxic and do not require alteration in the vascular bed to be effective. On the other hand, the bacterial polysaccharide appears to affect tumor cells only *in vivo*. Furthermore, it does affect the vascular system, and can lower the blood pressure as a delayed effect. The evidence does not point to the production of a sustained systemic hypotension as a necessary prerequisite for the polysaccharide to exert its antitumor effect. On the other hand, evidence is accumulating, from our own studies and from those of others on related phenomena, that the mechanism of the anti-tumor effect involves reactions in the blood and in the vascular bed.

References

(*1*) PRADHAN, S. N., ACHINSTEIN, B., and SHEAR, M. J.: Action of drugs of different pharmacological nature on the carotid blood pressure of the mouse. (Abstract.) J. Pharmacol. & Exper. Therap 113: 44, 1955.

(*2*) ———: Relation of the tumor-necrotizing effect of drugs to action on arterial blood pressure in mice. (Abstract.) Federation Proc. 14: 380, 1955.

(*3*) ALGIRE, G. E., LEGALLAIS, F. Y., and PARK, H. D.: Vascular reactions of normal and malignant tissues *in vivo*. II. The vascular reactions of normal and neoplastic tissues of mice to a bacterial polysaccharide from *Serratia marcescens* (*Bacillus prodigiosus*) culture filtrates. J. Nat. Cancer Inst. 8: 53–62, 1947.

(*4*) ALGIRE, G. E., and LEGALLAIS, F. Y.: Vascular reactions of normal and malignant tissues *in vivo*. IV. The effect of peripheral hypotension on transplanted tumors. J. Nat. Cancer Inst. 12: 399–421, 1951.

(*5*) ALGIRE, G. E., LEGALLAIS, F. Y., and ANDERSON, B. F.: Vascular reactions of the normal and malignant tissues *in vivo*. V. The role of hypotension in the action of a bacterial polysaccharide on tumors. J. Nat. Cancer Inst. 12: 1279–1295, 1952.

(*6*) ———: Vascular reaction of normal and malignant tissues *in vivo*. VI. The role of hypotension in the action of components of podophyllin on transplanted sarcomas. J. Nat. Cancer Inst. 14: 879–887, 1954.

(*7*) PRADHAN, S. N., ACHINSTEIN, B., and SHEAR, M. J.: Carotid blood pressure in normal and tumor-bearing mice. Am. J. Physiol. 184: 599–604, 1956.

(*8*) ———: Independence of drug-induced damage in Sarcoma 37 and carotid blood pressure in mice. Arch. exper. Path. u. Pharmakol. 230: 228–235, 1957.

(*9*) PERRAULT, A., and SHEAR, M. J.: The bacterial cell of *S. marcescens* as a source of tumor-necrotizing polysaccharide. Cancer Res. 9: 626–627, 1949.

(*10*) SHEAR, M. J., and PERRAULT, A.: Hemorrhagic necrosis in Sarcoma 37 induced with extracts of mammalian tissue. (Abstract.) Proc. Am. Assoc. Cancer Res. 1: 50–51, 1953.

(*11*) BELKIN, M., PERRAULT, A., and SHEAR, M. J.: Tumor-necrotizing polysaccharides from plant tissues. (Abstract.) Proc. Am. Assoc. Cancer Res. 2: 4, 1955.

(*12*) PERRAULT, A., and SHEAR, M. J.: Tumor-necrotizing polysaccharides from animal tissues. (Abstract.) Proc. Am. Assoc. Cancer Res. 2: 39–40, 1955.

(*13*) LEITER, J., DOWNING, V., HARTWELL, J. L., and SHEAR, M. J.: Damage induced in Sarcoma 37 with podophyllin, podophyllotoxin, alpha-peltatin, beta-peltatin, and quercetin. J. Nat. Cancer Inst. 10: 1273–1293, 1950.

(*14*) SCHRECKER, A. W., GREENBERG, G. Y., and HARTWELL, J. L.: Components of podophyllin. XVI. Podophyllotoxin haloacetates and quaternary derivatives. (Abstract.) J. Am. Chem Soc. 76: 1184–1185, 1954.

(*15*) LEITER, J., DOWNING, V., HARTWELL, J. L., and SHEAR, M. J.: Damage induced in Sarcoma 37 with chemical agents. III. Colchicine deratives related to trimethylcolchicinic acid and to colchinol. J. Nat. Cancer Inst. 13: 379–392, 1952.

(*16*) LEITER, J., HARTWELL, J. L., KLINE, I., NADKARNI, M. V., and SHEAR, M. J.: Damage induced in Sarcoma 37 with chemical agents. IV. Derivatives of colchiceinamide. J. Nat. Cancer Inst. 13: 731–739, 1952.

(*17*) HARTWELL, J. L., LEITER, J., PERRAULT, A., and SHEAR, M. J.: Unpublished data.

(*18*) SHEAR, M. J., PERRAULT, A., and ADAMS, J. R., JR.: Chemical treatment of tumors. VI. Method employed in determining the potency of hemorrhage-producing bacterial preparations. J. Nat. Cancer Inst. 4: 99–105, 1943.

(*19*) STILL, J. W., PRADHAN, S. N., and WHITCOMB, E. R.: Direct measurements of aortic blood pressure in unanesthetized rats. J. Appl. Physiol. 8: 575–576, 1956.

(*20*) BRUES, A. M., and SHEAR, M. J.: Chemical treatment of tumors. X. Reactions of four patients with advanced malignant tumors to injection of a polysaccharide from *Serratia marcescens* culture filtrate. J. Nat. Cancer Inst. 5: 195–208, 1944.

(*21*) SHEAR, M. J. *et al.*: Some aspects of a joint institutional research program on chemotherapy of cancer: current laboratory and clinical experiments with bacterial polysaccharide and with synthetic organic compounds. *In* Approaches To Tumor Chemotherapy. Washington, D. C., Am. Assoc. Advancement Sc., 1947, pp. 236–284.

(*22*) PRADHAN, S. N., ACHINSTEIN, B., and SHEAR, M. J.: Effect of various drugs on the tumor-necrotizing activity of several chemical agents in mice. Cancer Res. 16: 1062–1068, 1956.

Growth of Pulmonary Tumors in Mice of Strains A and C3H [1]

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Summary

Quantitative measurements were carried out on the growth of induced primary pulmonary tumors in mice of strains A and C3H. The growth rate characteristic of the tumors appears to be a property of the tumor itself and particularly dependent upon the size of the mass. The growth rate of pulmonary tumors is more rapid in strain A than in strain C3H mice by a ratio of 1 to 0.6. The growth is manifested by a rapid initial phase with gradual deceleration. It is suggested that this deceleration may be due in part to a negative gradient of the rate of cell division from the surface toward the center of the tumor mass. **J. Nat. Cancer Inst. 21: 595-610, 1958.**

In a previous report (*1*) data were presented on the induction and growth of primary adenomatous pulmonary tumors in strain A mice that received an injection of urethan. The growth of the tumors was followed for 19 weeks. When the mean size of the tumors was plotted against time, on semilogarithmic paper, the points fell on a curve of gradually diminishing slope indicating a gradually decreasing specific rate of growth.

The investigation is now extended to include a longer period of observation, 52 weeks, and to explore the influence of dose, type of carcinogen, and strain of mouse upon the growth of the tumors. The chief purpose is to seek further insight into the nature of the growth, especially the mechanism of deceleration of the growth rate of the tumors.

Materials and Methods

Mice of strains A and C3H at the age of 1.5 to 3 months were used. Ethyl carbamate (urethan) as a 10 percent aqueous solution was injected intraperitoneally. Single doses of 0.1, 0.25, and 1.0 mg. per gm. of body

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weight were given to strain A mice. C3H strain mice received 1.0 mg. per gm. body weight in 1 to 5 daily doses, for an average dose of 3.5 mg. per gm. body weight.

Lung sections obtained from Heston were also used. In his unreported and reported experiments (*2*, *3*) mice received nitrogen mustard [methyl-bis(β-chloroethyl)amine hydrochloride,[4] hereinafter referred to as HN_2] and 1,2,5,6-dibenzanthracene [5] (hereinafter referred to as DBA). HN_2 was dissolved in water, 1 mg. in 100 cc., and given intravenously in 2 doses, 2 days apart, 0.001 mg. per gm. of body weight, for a total dose of 0.04 to 0.05 mg. per animal. DBA was dispersed in horse serum, 4 mg. in 100 cc., and injected intravenously in a single dose, 0.02 mg. per animal.

After periods of time following the administration of the carcinogens, as specified in table 1, groups of mice were killed, and their lungs excised and fixed. The nodules visible on the surface were counted, and the right major lobe of the lung was serially sectioned. When the number of tumors was small, as in the C3H mice, nodules visible in other areas of the lung were added to the right lobe specimen and these also were serially sectioned. All sections were stained with hematoxylin and eosin.

When an adenomatous pulmonary tumor was found under the microscope, contiguous sections were examined and the largest of the areas (*i.e.*, the "equatorial" area of the approximately spheroid structure) was taken as the direct measure of the size of the tumor. This area was oriented into a Whipple micrometer introduced into the eyepiece of the microscope and drawn on a grid that was a magnified reproduction of the micrometer grid. The drawn area was then measured, with appropriate adjustment for magnification.

Since the diameter of a tumor cell is of the order of 10 microns, we have chosen to express lengths, areas, and volumes in terms of a special unit of length designated a *cellular unit*. A cellular unit is defined as 10 microns. Thus, a tumor with an area of 10,000 square cellular units shows approximately 10,000 cells in its equatorial section; the diameter is of the order of 100 cell diameters.

Most pulmonary tumors in mice are approximately spheroidal. The growth of a tumor may be followed by observing the change in diameter, in equatorial area, or in volume. The *area*, *i.e.*, the quantity measured directly, was chosen as the measure of the growth of the tumors. For reasons previously given (*1*), the *geometric mean* of the areas rather than the arithmetic mean was selected to express the average values of the tumor samples.

The gross and histologic morphology of primary pulmonary tumors in mice is rather monotonous in its regularity and similarity (*1*). In the previous report, photomicrographs were shown of representative tumors in strain A mice killed at 21 to 133 days following urethan. In this experiment, tumors in strain A mice killed up to 365 days after urethan and in

[4] *Chemical Abstracts'* nomenclature: 2,2'-dichloro-*N*-methyldiethylamine hydrochloride.
[5] *Chemical Abstracts'* nomenclature: dibenz [*a*,*h*] anthracene.

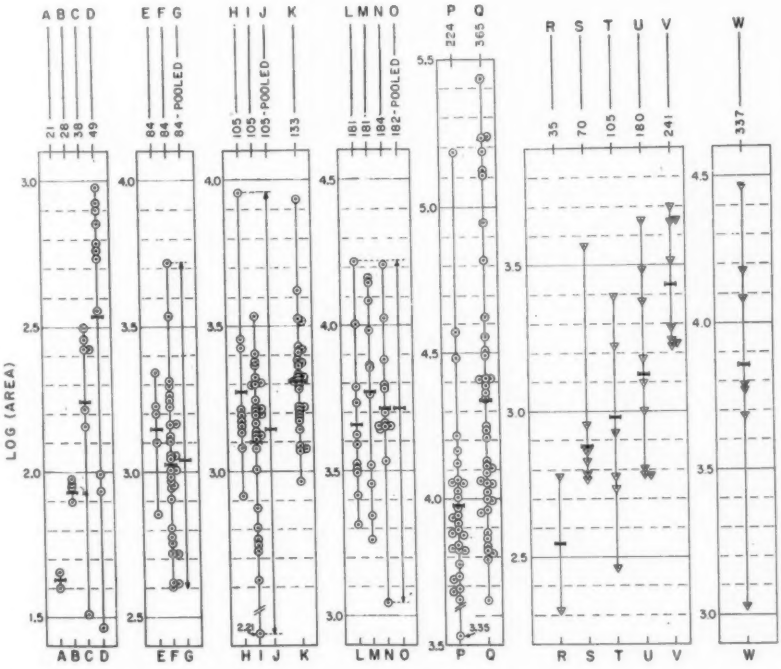
C3H mice killed up to 337 days after urethan were of the same cellular appearance.

There were no clear morphologic differences between the earliest recognizable tumors and the later, larger tumors. Specifically, none of the larger tumors demonstrated central necrosis. There were examples of coalescence of tumors, but on section it was usually not difficult to find a definite line dividing the individual tumors at the collision.

Results

Table 1 presents the essential data on the 20 groups of mice used in the experiment. Included are measurements of 227 lung tumors elicited in 123 mice of strain A and 38 lung tumors from 110 mice of strain C3H killed from 21 to 365 days after injection of the carcinogens.

Text-figure 1 gives the log area measurements for each of the tumors. Groups G, J, and O are pooled samples from strain A mice killed at approximately the same time following the experimental procedure.



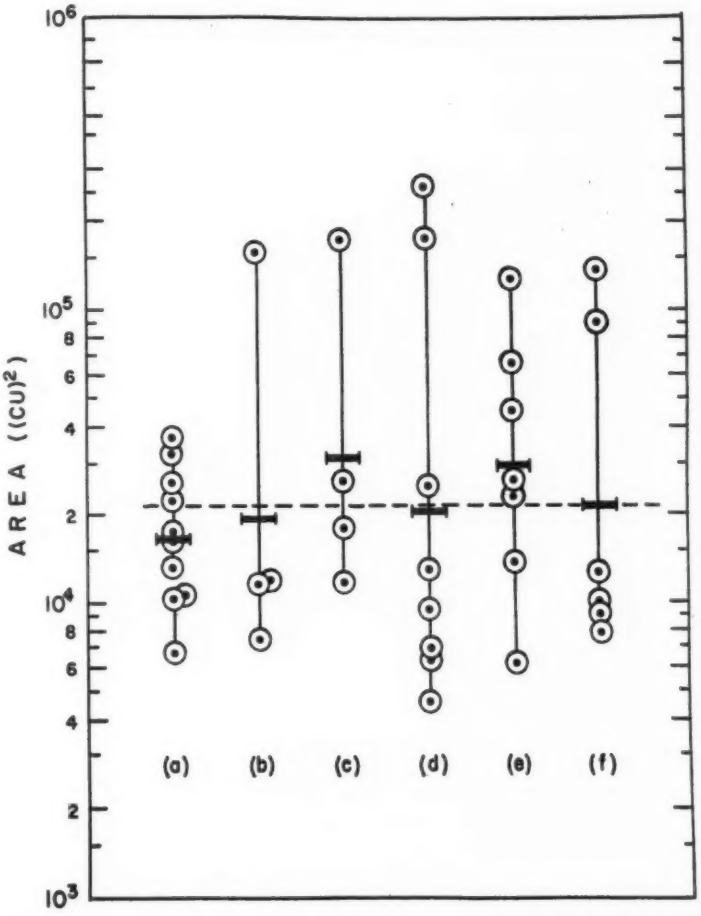
TEXT-FIGURE 1.—Distribution of sizes of pulmonary tumors in mice. The ordinate of each point represents the logarithm of the "equatorial" area of the respective tumor expressed in square cellular units. Numbers at *top* of each panel indicate the time, in days, after injection of carcinogen. Horizontal bar in *panels* indicates the mean of log areas. Group A through Q are strain A mouse tumors, represented by circles. R through W are strain C3H mouse tumors, represented by triangles.

TABLE 1.—*Summary of data on pulmonary tumors in mice*

Group	Mice		Carcinogen	Time (*days*)	Mean surface tumors per animal (*number*)	Tumors measured (*number*)	Geometric mean of area		Standard deviation of log $\bar{A}$
	Strain	Number					$\bar{A}$	Log $\bar{A}$	
							cu[2]	*cu*[2]	
A	A	20	1 mg. urethan/gm.	21	—	2	43	1. 627	—
B	"	15	"	28	0. 27	4	88	1. 944	—
C	"	5	"	38	0. 80	7	174	2. 240	—
D	"	10	"	49	2. 7	11	314	2. 536	—
E	"	10	0.25 mg. urethan/gm.	84	1. 9	5	1, 390	3. 144	0. 185
F	"	10	1.00 mg. urethan/gm.	84	11. 2	29	1, 055	3. 023	0. 269
(G)	"	—	(pooled E and F)	(84)	—	(34)	1, 100	3. 041	0. 259
H	"	10	0.25 mg. urethan/gm.	105	4. 4	10	1, 865	3. 270	0. 286
I	"	5	1.0 mg. urethan/gm.	105	17. 0	28	1, 247	3. 096	0. 288
(J)	"	—	(pooled H and I)	(105)	—	(38)	1, 390	3. 142	0. 294
K	"	5	1 mg. urethan/gm.	133	15. 4	28	2, 090	3. 311	0. 193
L	"	6	0.05 mg. HN_3	181	3. 1	11	4, 550	3. 658	0. 268
M	"	6	0.1 mg. urethan/gm.	181	1. 8	11	5, 860	3. 768	0. 324
N	"	8	0.02 mg. DBA	184	3. 4	13	5, 140	3. 711	0. 270
(O)	"	—	(pooled L, M, and N)	(182)	—	(35)	5, 150	3. 712	0. 283
P	"	7	1 mg. urethan/gm.	224	28. 6	29	9, 455	3. 976	0. 335
Q	"	6	"	365	26. 5	39	21, 160	4. 334	0. 475
R	C3H	20	1-5 mg. urethan/gm.	35	0. 1	2	352	2. 546	—
S	"	20	"	70	0. 3	6	910	2. 959	0. 305
T	"	19	"	105	0. 3	6	832	2. 920	0. 341
U	"	28	"	180	0. 3	9	1, 340	3. 127	0. 322
V	"	14	"	241	0. 6	8	2, 730	3. 436	0. 212
W	"	9	"	337	0. 8	7	7, 190	3. 856	0. 456

Variation of tumor size within experimental groups.—Text-figure 1 shows that there is, generally, a large variation in area size within the groups, both in A tumors and in C3H tumors. The greatest variations occur in groups P and Q where the largest areas are some 45 times larger than the smallest areas. The ratio of the corresponding volumes is approximately 300.

It is of interest to determine how much of the variation of sizes within a group is due to a possible difference in the rate of growth among the animals from which the tumors were taken. Text-figure 2 shows the

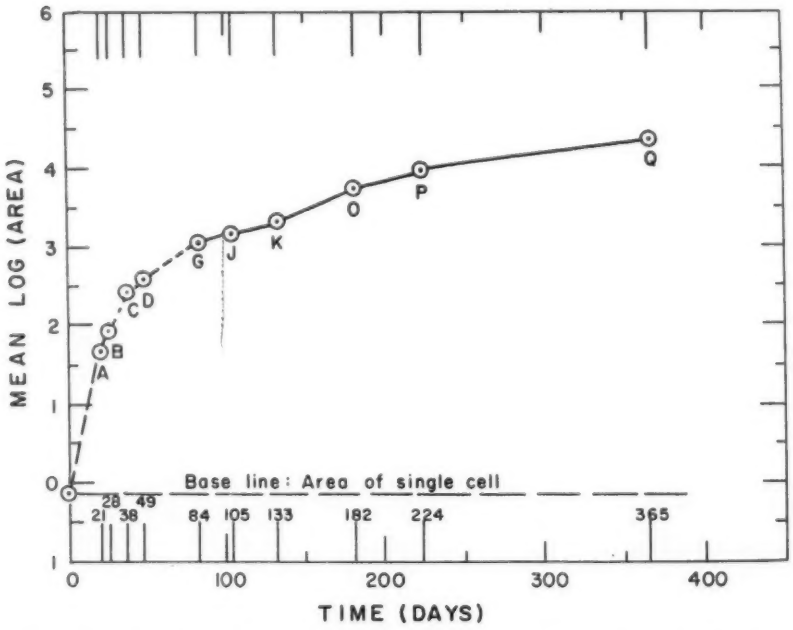


TEXT-FIGURE 2.—Distribution of tumor sizes in the right major lung lobe of 6 strain A mice 365 days following 1 mg. of urethan per gm. body weight (group Q). The mice are identified (a) to (f). The horizontal bars indicate the mean log area of the respective tumor sample; the mean of the pooled total of all tumors is indicated by the dashed horizontal line.

sizes of the 39 tumors of group Q classified according to the 6 individual animals from which the tumors were taken. The text-figure indicates that the major source of variation is in the size of individual tumors rather than in the individual mice of the group.

Effect of dose of urethan on rate of growth.—Tumor groups E, F and H, I were taken from strain A mice receiving 0.25 mg. and 1.0 mg. urethan per gram, respectively. As may be seen in text-figure 1, the mean was slightly higher for the smaller dose in each of the two pairs. An F test on the significance of the differences of the means in the two pairs indicated that in each pair the difference is significant at approximately the 5 percent level. However, the absolute differences are rather small when viewed on the scale of text-figure 3, and for this reason the pairs E, F and H, I, respectively, were pooled for further analysis.

Effect of type of carcinogen upon rate of growth.—Groups L, M, and N were taken from animals receiving HN_2, urethan, and DBA, respectively. The horizontal bars along lines L, M, and N of text-figure 1 show the 3 means. An F test of the significance of the differences led to the tentative conclusion that there is no statistically significant difference between the 3 means. Accordingly, the 3 samples were pooled for further analysis.



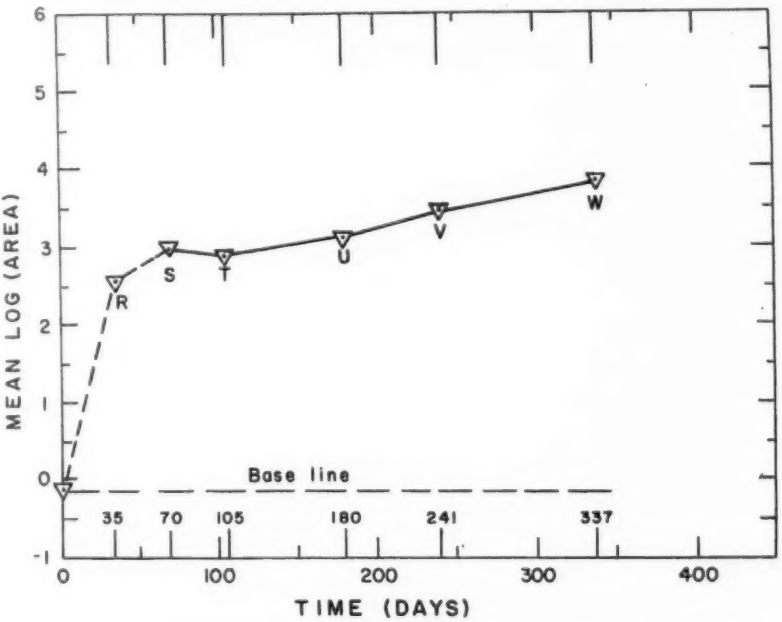
TEXT-FIGURE 3.—The growth of induced pulmonary tumors in strain A mice. The dashed line joining point A and the point of zero time shows the growth from a single cell and presumes no latent period. Line D–G is dashed since not all tumors are recognized by 49 days.

Growth of strain A tumors.—Text-figure 3 shows the progress, with time, of the size of strain A tumors. The point at zero time represents the size of the "initial tumor," namely, the mean area of a single tumor cell. The mean volume of a tumor cell is 0.5 cu^3 (*1*). The equatorial area of a sphere of this size is 0.76 cu^2; its logarithm is −0.119.

As may be seen from text-figure 3, the deceleration of the specific rate of growth continues up to the maximum age of tumor groups examined, namely, 365 days.

It is interesting to note that the two sets of points A to K and O to Q, whose measurements were made in San Francisco and in Bethesda several years apart, fall on a single curve.

Growth of strain C3H tumors.—Text-figure 4 shows the progress, with time, of the size of C3H tumors. This strain of mice is a great deal more



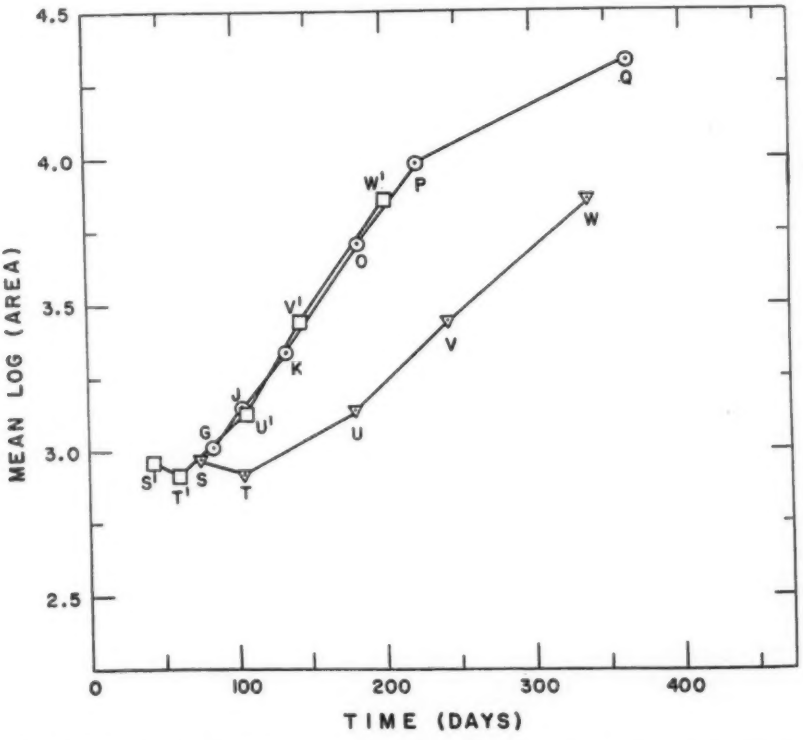
TEXT-FIGURE 4.—The growth of induced pulmonary tumors in strain C3H mice. As in text-figure 3, the dashed line to the base line of a single cell is shown under the assumption that individual tumors arise from single cells and that there is no appreciable latent period between the administration of the carcinogen and the induction of the tumor.

resistant to induction of lung tumors than strain A. The same dose of urethan induces approximately 30 times as many tumors in strain A as in strain C3H. Thus, even though tumors were taken from the entire lungs of the C3H mice, the number of tumors in the C3H tumor samples was smaller than the number of tumors taken from single lobes of strain A animals.

If the curve begins with the area of a single cell, it is obvious from the figure that the specific rate of growth is a decelerating quantity during the early part of the process of growth. The tumor samples were not large enough to yield reliable information as to whether the specific rate beyond 100 days continues to decelerate or whether it remains constant. On the other hand, there is sufficient information to compare the growth of tumors of the two strains.

Comparison of growth rate of tumors in strains A and C3H.—Text-figure 5 presents the growth curves for both strain A tumors and strain C3H tumors. When two rates of growth are exponential, it is a simple matter to compare the two rate constants since their ratio is merely the ratio of the slopes of the two curves. When the growth curve is more complex, this method of comparison is not feasible.

Let it be assumed, however, that the process of growth is of such a nature that the instantaneous specific rate of change of volume is the



TEXT-FIGURE 5.—Comparison of rates of growth of induced pulmonary tumors in strain A and strain C3H mice. The mean tumor areas of strain A mice are represented by circles and that of C3H mice by triangles. The letters by the individual points refer to specific groups as detailed in text-figure 1 and table 1. The squares marked with letters S,′ T,′ U,′ V,′ and W′ are values derived by applying a factor of 0.6 to each abscissa of the series S, T, U, V, W.

product of a rate constant whose value changes from one tumor to another and of a tumor-size factor which has the same function of tumor size for all tumors that are being compared. If V is the instantaneous volume of the tumor, this relation may be expressed in the following mathematical form:

$$\text{Instantaneous specific rate} = \frac{1}{V}\frac{dV}{dt} = kf(V) \qquad [1].$$

The solution of equation [1] is of the type:

$$G(V) - G(V_o) = kt \qquad [2].$$

where V_o is the initial volume of the tumor, *i. e.*, the volume of a single cell.

Equation [2] indicates that the curves corresponding to two different values of k differ only in the time scale. Two tumors whose rate constants are k_1 and k_2, respectively, achieve equal volumes at times t_1 and t_2, whose relative values are given by the relation : $t_2 = t_1k_1/k_2$. It follows that the two volume curves can be made to coincide if each of the abscissas of the second curve is multiplied by the factor k_2/k_1. The same relation holds true between the curves of equatorial areas plotted against time or between the curves of any function of V plotted against time.

Given two populations of tumors, A and B, each possessing a distribution of values of k, then, with the added assumption that the standard deviation of log k is the same in the two populations, it may be asserted that the two curves of the means of V may be made to coincide by a change of the time scale of one of the curves of means.

Given the two curves of means, we may detect the presence of a difference of time scale only by plotting $\overline{V}$ or any function of $\overline{V}$, such as $\overline{\log V}$ or $\overline{\log A}$, against the logarithm of time. If the time scale is the only difference between the two growth curves, the two curves will be parallel, separated by a horizontal displacement of log $(\overline{k}_A/\overline{k}_B)$. Alternatively, multiplication of all the time values of curve B by the factor $\overline{k}_B/\overline{k}_A$ should bring about the transformation of curve B into curve A.

A comparison of the rates of growth of strain A and strain C3H tumors is presented in text-figure 5. The mean log values of the areas of strain A and strain C3H tumors are shown along with a curve obtained by multiplying each abscissa of the C3H curve by the factor 0.60. The coincidence of the transformed curve with the strain A curve suggests that the mean rate constant for C3H tumors is smaller than the mean rate constant for strain A tumors by a factor of 0.60. If follows that the doubling time for strain A tumors, for a given size of tumor, is smaller than the doubling time for C3H tumors by the same factor.

Nature of the growth curve.—When the mean equatorial areas are plotted against time on a semilogarithmic field, the instantaneous slope at any time yields the value of the specific rate of change, as may be seen from the following equation:

$$\frac{d \log \overline{A}}{dt} = \frac{1}{\overline{A}}\frac{d\overline{A}}{dt} \qquad [3].$$

The instantaneous specific rate of change of volume is larger than the specific rate of change of equatorial area by the factor 3/2, as may be seen from the following equation:

$$\overline{V} = \text{constant} \times \overline{A}^{3/2}; \log \overline{V} = \log (\text{constant}) + 1.5 \log \overline{A}$$

$$\frac{d \log \overline{V}}{dt} = \frac{1.5\, d \log \overline{A}}{dt} \qquad [4].$$

The same relation implies that the instantaneous doubling time of the volume or of the total number of cells in the tumor equals two thirds of the doubling time of the equatorial area.

The experimental results show that the specific rate decreases as the mean tumor size increases. The results with strain A tumors suggest that the deceleration continues over the entire measuring period, namely, 365 days. The question arises as to the mechanism causing the deceleration of specific rate.

It is probably naïve to expect that the complex process of tumor growth can be interpreted by the use of a simple model. Nevertheless, the development of such models and examination for concordance with the observed data are useful.

Several theoretical models were considered in interpreting the growth-rate curves discussed in the previous paper (*1*). The extended data, available through additional observations, have made it possible to deal in more detail with the model which assumes that the deceleration of the specific rate of growth is associated solely with the increasing size of the tumor. It may be that there is decrease in the diffusion and availability of nutrients to the cells in the interior of the tumor mass, with resultant lack of division except in cells lying close to the periphery of the mass. In this model, starting with the outermost layers of cells, there is a negative gradient of cell division in the direction toward the center of the tumor. Assuming this model, it is possible to estimate from the experimental results the magnitude of the gradient.

For large tumors it may be assumed, as a first approximation, that the specific rate decreases exponentially with the distance from the surface of the tumor. Let R be the radius of the tumor; let a be the distance from the surface at which the local specific volume rate has dropped from the value k at the surface to the value ke^{-1}. In that case the local specific volume rate at a distance r from the center is $ke^{-(R-r)/a}$.

The instantaneous rate of growth of a tumor of radius R is given by the following equation:

$$\text{Instantaneous rate} = \frac{dV}{dt} = \int_0^R ke^{-(R-r)/a} 4\pi r^2 dr$$

$$= 4\pi R^2 ka \left[1 - 2\frac{a}{R} + 2\left(\frac{a}{R}\right)^2 - 2\left(\frac{a}{R}\right)^2 e - \frac{R}{a}\right] \qquad [5].$$

To anticipate later results, it may be stated that the value of a is between 2 and 3 cellular units. On the other hand, the values of R for the

tumors to be tested by this equation are greater than 10 cellular units. It follows that the last term within the brackets is of negligible magnitude. Equation [5] may be replaced by equation [6]:

$$\text{Instantaneous rate} = dV/dt = 4\pi R^2 ka\left[1 - 2\frac{a}{R} + 2\left(\frac{a}{R}\right)^2\right] \qquad [6].$$

The instantaneous rate of change of the radius is given by the following equation:

$$\frac{dR}{dt} = \frac{dR}{dV}\frac{dV}{dt} = \frac{1}{4\pi R^2}\frac{dV}{dt} = ka\left[1 - 2\frac{a}{R} + 2\left(\frac{a}{R}\right)^2\right] \qquad [7].$$

Equation [7] is similar to the equation yielded by a model of tumor growth proposed by Mayneord (*4*). In Mayneord's model the growth of a spherical tumor is confined to an outer thin shell of thickness a. Within the shell the specific rate is constant, whereas the specific rate is zero within the inner core. Mayneord's model yields the following equation for the instantaneous rate of growth of the radius:

$$\frac{dR}{dt} = ka\left[1 - \frac{a}{R} + \frac{1}{3}\left(\frac{a}{R}\right)^2\right] \qquad [8].$$

Both equation [7] and [8] predict that, at very high values of R/a, the radius will increase at the constant rate ka. When the value of R/a is 10, the rates predicted by the two equations differ by approximately 10 percent. When $R/a = 30$, they differ by approximately 3.3 percent.

The value of ka of equation [7] may be obtained by plotting the radius against the age of the tumor. If the model is correct, the points will fall on a straight line, and the value of ka will be given by the slope of the line.

The value of a, *i.e.*, the thickness over which the activity drops by a factor of e^{-1}, may be obtained from the value of ka as follows: When the tumor is very small, then in the model under discussion the diffusion effect is almost negligible. Let the instantaneous values of the volume and equatorial area be V and A, respectively, then the following relations hold:

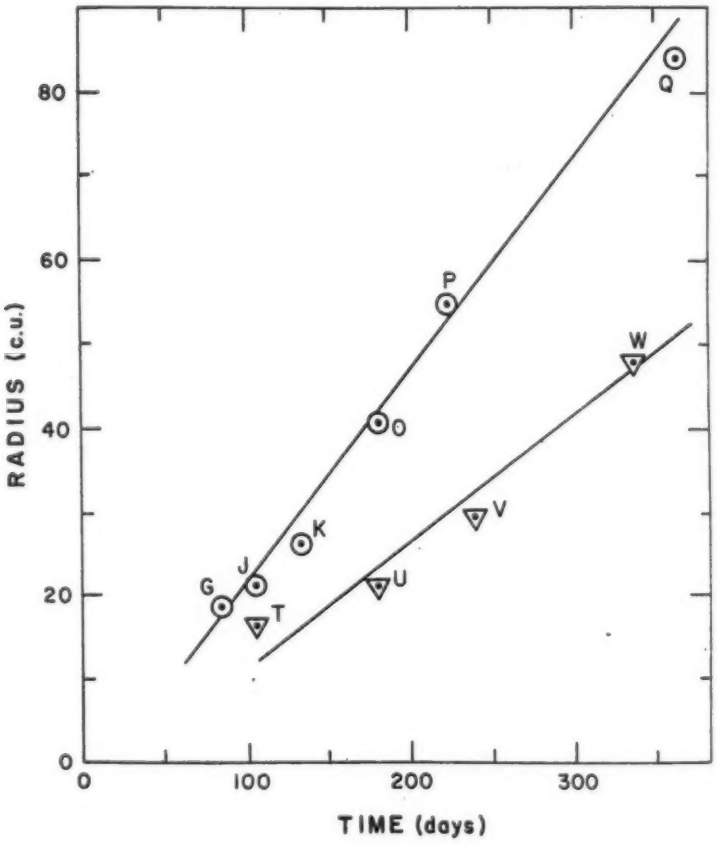
$$\frac{dV}{dt} = kV, k = \frac{dV}{Vdt} = \frac{dlnV}{dt} \qquad [9a].$$

$$lnV = \text{constant} + \frac{3}{2}\,ln\,A \qquad [9b].$$

$$k = \frac{dlnV}{dt} = \frac{3}{2}\frac{dlnA}{dt} = \frac{3}{2} \times 2.303\frac{d\log A}{dt} = 3.45\frac{d\log A}{dt} \qquad [9c].$$

Thus, an estimate of the value of k may be obtained from the measurements of very small tumors. This value, divided into the constant rate ka of old tumors, will yield an estimate of a.

A plot of the values of the mean radius of the tumors against time is shown in text-figure 6. The slope of the straight line for strain A tumors is 0.25 cellular units per day. The slope for C3H tumors is 0.15 cellular units per day. As shown earlier, the slope of the line equals the product ka, and to evaluate a it is necessary to estimate the value of k for each of the two strains from data obtained with small tumors.



TEXT-FIGURE 6.—Plot of the geometric means of the radii of tumor groups against the age of the group. The linear relationship suggests that there is a rapid decrease of the rate of cell proliferation from the surface of the tumor toward its center (*see* text).

According to equation [9c], $k = 3.45\ d\log A/dt$. The derivative is to be evaluated at very low values of t when the tumors are very small. Since the nature of the experiments does not allow the determination of tumor sizes when the tumors are very small, it will be necessary to give an estimate of the value of k in the form of an inequality:

From the growth curve shown in text-figure 3, it is clear that the early values of $d\log\overline{A}/dt$ are smaller than the slope of the line drawn from the point at zero time, representing a "tumor" consisting of a single cell, to point G. The logarithm of the equatorial area of a single cell is -0.12. The value of $\log\overline{A}$ at point G is 3.041 (table 1). The slope of the line oG is, therefore, 0.038 day^{-1}. It follows that the value of the slope is larger than 0.038 day^{-1}.

Using the same method for strain C3H tumors and the data for point T of text-figure 4 and table 1, we may conclude that for this strain the value of the slope is larger than 0.029 day^{-1}.

With these inequalities, the following statements may be made about the values of a for the two strains:

For strain A tumors:

$ka = 0.25$; $k > 3.45 \times 0.038 = 0.136$ day^{-1}; $a < 0.25/0.136 = 1.84$ cu.

For strain C3H tumors:

$ka = 0.15$; $k > 3.45 \times 0.029 = 0.10$ day^{-1}; $a < 0.15/0.10 = 1.50$ cu.

The meaning of the various quantities calculated for the activity gradient model may be illustrated by the following calculations applied to strain A tumors:

The inequality $k > 0.136$ day^{-1} is independent of the nature of the model. The doubling time T_2 is given by the equation: $e^{kT_2} = 2$; $T_2 < 0.69/0.136 = 5.1$ days. The doubling time is less than 5.1 days.

Large tumors increase at a constant rate $d\overline{R}/dt = ka = 0.25$ cu per day. From the volume of a single cancer cell (0.5 cu^3), it may be calculated that the cellular diameter is 0.98 cu; it follows that the mean rate of increase of the tumors, when they are large, is approximately one layer of cells every 4 days.

The quantity a was defined as the distance from the surface over which the mean specific rate decreases by the factor $e^{-1} = 1/2.7$. Since the diameter of a cell is 0.98 cu, the outermost layer of cells is at a mean distance of 0.49 cu from the surface. The second layer is at a mean distance of 1.47 cu from the surface. If the mean volume specific rate of growth of an unencumbered cell is k, the mean specific rates of growth of successive layers of cells in a large tumor are given by the following values:

Layer	1st	2nd	3rd	4th	6th
Mean distance from surface$=h$	0.49	1.47	2.45	3.43	5.39 cu
Relative specific rate$= e^{-h/a}$	<0.76	<0.45	<0.26	<0.16	<0.053

These calculations indicate that, if the gradient of activity model is correct, there is a sharp gradient of activity from the surface inward. The sixth layer of cells possesses a specific growth rate which is less than 5 percent of the rate of an unencumbered cell.

The equivalent fraction of active cells may be estimated in the following manner: In a tumor where all the cells multiply at the specific rate k, the rate of increase of volume is given by the following relation: $dV/dt = kV = \frac{4}{3}\pi R^3 k$. In the model under consideration the rate of change of volume is $\frac{4}{3}\pi R^2 dR/dt = \frac{4}{3}\pi R^2 ka$. In other words, the observed rate of change of volume is smaller than the rate in a freely growing tumor in the ratio a/R. In tumor group Q, where the mean radius was 84 cu, the value of a was smaller than 1.84 cu. Thus, the mean rate of growth of the tumor was the same as if only the fraction $p < 1.84/84$, or less than 2.2 percent of the total number of cells, multiplied at the specific rate k.

Discussion

The growth-rate curve for induced pulmonary tumors in strain A mice was derived from findings of at least 6 separate experiments performed in San Francisco and in Bethesda over a period of at least 4 years. There is a wide variability in the size reached by individual tumors and therefore in their growth rate, yet the reproducibility of the mean growth of these induced tumors is surprisingly uniform. This reproductibility holds whether groups of animals or individual animals are considered.

Results of the experiments support the view that the growth of neoplasms is independent of the initiating carcinogen. Thus, the growth rate is approximately the same whether small or large doses of the same carcinogen (urethan) or different carcinogens (urethan, dibenzanthracene, or nitrogen mustard) are used. Since the number of individual tumors per lung is proportional to the dose of the carcinogen, this proportion leads to the conclusion that there is no interaction between the growth of these separate tumors. That is, the growth rate is independent of the number of separate foci of tumor evoked.

Comparison of the growth rate of pulmonary tumors in the highly susceptible strain A and the more resistant strain C3H shows that the pulmonary tumors grow more slowly in the latter strain. The ratio of the rates of growth of pulmonary tumors in strains A and C3H is approximately 1 to 0.6. This difference between the two strains is markedly less than the difference in response as measured by the number of separate tumors evoked by equal doses of urethan, in which case A mice demonstrate some 30 times the number of tumors seen in C3H mice. Thus, susceptibility to neoplastic initiation in these strains is of a greater order of difference than the subsequent rates of growth of the tumors. However, some difference in the growth rate is manifested, due either to the inherent growth-rate differences in the tumor-cell population or to the resistance factors of the host.

In this connection we had the opportunity to measure some tumors evoked in lung transplants, reported by Heston and Dunn (*5*). Lung tissue of strain A and strain C57L mice was transplanted subcutaneously into hybrids of these two strains. The LA F_1 mice were injected intra-

venously with dibenzanthracene, and lung tumors were induced in the transplanted tissue. The 15 lung tumors that arose in strain A tissue were larger than the 3 tumors originating in C57L lung transplants. This difference in size indicates that the relative rate of growth is a characteristic of the tissue from which the tumor is derived rather than being under the control of the host. However, the strain A tumors in subcutaneous transplants were smaller than the tumors in the lung after a comparable time period, indicating the influence of environmental factors associated with transplantation.

A common characteristic of all pulmonary-tumor growth rates recorded here is the rapid initial phase and the gradual deceleration of growth at later time periods. This deceleration begins when the tumors are quite small.

One theoretical mechanism of this deceleration is that the environmental factors necessary for cell division are less available to the cells in the interior of the tumor mass than those at the periphery, resulting in a negative gradient of the rate of cell division in the direction from the surface toward the center of the tumor mass.

A mathematical model of this premise was tested previously (*1*) and the following conclusion was reached: "... it should be mentioned that the theoretical course discussed by Mayneord will not fit [the] curve.... In Mayneord's model, as the sphere becomes large, growth begins to be limited to an outer layer of the sphere, and the rate of change of the radius approaches a constant value."

The extension of the observation over a longer period of time allows revision of this view. The experimental points indeed do fall approximately upon a straight line. It is concluded, therefore, that the deceleration of the growth rate of these organoid neoplasms may be due, at least in part, to different rates of growth of cells in their relation to the total mass, with only a relatively few layers of the cells toward the surface being actively in proliferation.

The possible relevance of these observations to other problems of tumor growth, particularly the growth of tumors in man (*6*), is of some practical importance. The growth of tumors is usually measured when the tumors are considerably larger than the pulmonary tumors of mice, and usually the time of initiation is unknown. The extrapolation backward of the observed growth rate toward a theoretical time of initiation becomes most hazardous if the deceleration effect observed in pulmonary tumors in mice also takes place in human tumors.

References

(*1*) SHIMKIN, M. B., and POLISSAR, M. J.: Some quantitative observations on the induction and growth of primary pulmonary tumors in strain A mice receiving urethan. J. Nat. Cancer Inst. 16: 75–97, 1955.

(*2*) HESTON, W. E.: Induction of pulmonary tumors in strain A mice with methyl-bis(β-chloroethyl)amine hydrochloride. J. Nat. Cancer Inst. 10: 125–130, 1949.

(*3*) HESTON, W. E., and SCHNEIDERMAN, M. A.: Analysis of dose-response in relation to mechanism of pulmonary tumor induction in mice. Science 117: 109–111, 1953.

(4) MAYNEORD, W. V.: On a law of growth of Jensen's rat sarcoma. Am. J. Cancer 16: 841–846, 1932.

(5) HESTON, W. E., and DUNN, T. B.: Tumor development in susceptible strain A and resistant strain L lung transplants in LA F_1 hosts. J. Nat. Cancer Inst. 11: 1057–1171, 1951.

(6) COLLINS, V. P., LOEFFLER, R. K., and TIVEY, H.: Observations on growth rates of human tumors. Am. J. Roentgenol. 76: 988–1000, 1956.

Effect of Lack of Glutamine on Subline 2071 Mouse Cells [1]

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Summary

Cells of the subline NCTC 2071 from NCTC clone 929 of the L strain of cells of mouse fibroblast origin were grown continuously in synthetic medium NCTC 109 for 30 months. The medium contained no added protein. Since this is the only known strain of cells so far adapted to growth in synthetic protein-free medium and grown continuously for long intervals, its nutritional requirements were examined in detail. This was done both to simplify the medium and to get information that might be useful in adjusting other long-established cell strains to growth in the protein-free nutrient fluid. Three experiments were set up: (A) the control on the stock NCTC 109 protein-free medium, (B) the same medium without glutamine, and (C) the same medium with glutamine and asparagine omitted. In all instances the population numbers fell below the inoculum numbers in the absence of glutamine. The drop in populations was usually precipitous and could be seen in some instances within 17 hours after the cells were transferred to the deficient medium. Glutamine was very rapidly removed from the medium by the cells when it was present. The few surviving cells in the (B) and (C) mediums apparently obtained enough glutamine by the destruction of their neighbors to function metabolically to a limited extent. **J. Nat. Cancer Inst. 21: 611-620, 1956.**

A number of studies showed (*1–10*) that glutamine was a necessary growth factor for mammalian cells in tissue culture.[3] As part of such work, growth studies were done on the mouse parenchymal liver cells (*11*) in the presence and absence of glutamine. Extensive studies were made on the disappearance of glucose from the medium simultaneously with the production of lactic acid under these conditions. Changes in concentration of various amino acids and of pyruvic acid, α-ketoglutaric acid,

[1] Received for publication April 25, 1958.

[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

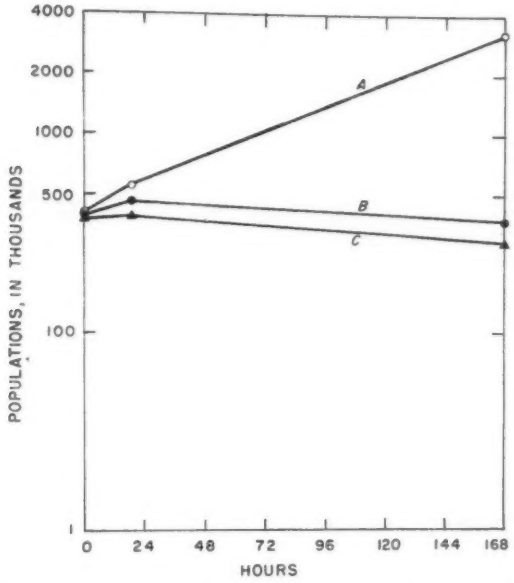
[3] Actually, the work reported in reference *6* indicates that freshly explanted chick embryonic heart fibroblasts do not need added glutamine.

and glutamine were followed. The mouse liver strains, in the absence of glutamine, did not maintain the inoculum numbers and could not use glucose. They used very limited amounts of amino acids; they did not produce and return to the medium lactic, pyruvic, or α-ketoglutaric acids.

It was demonstrated recently (*12*) that more nearly definitive data on effects of amino acids could be obtained by the use of NCTC 2071 cells in the NCTC 109 medium in a shorter time than when NCTC clone 929 cells of strain L were used. It appeared that the results formerly found by Sanford *et al.* (*13*) on glutamine utilization could be even more critically defined under the conditions used in the present study. The NCTC 2071 cells had been on the protein-free medium for 30 months, whereas the NCTC clone 929 of L strain had been maintained in undefined stock medium of horse serum and chick-embryo extract for 17 years.

Materials and Methods

Replicate cultures (*14*) of subline NCTC 2071 (*15*) of NCTC clone 929 (strain L) cells were prepared in complete NCTC 109 medium, NCTC 109 with glutamine omitted from the amino acid mixture, and NCTC 109 with both glutamine and asparagine omitted from the medium. Enumeration of nuclei (*16*) provided the population of the original inoculums. The cells were maintained for 17 hours with no fluid change, 7 days with one fluid change at the end of 3 days, and in one experiment, extending through 14 days, fluid renewals were made at the 3rd,



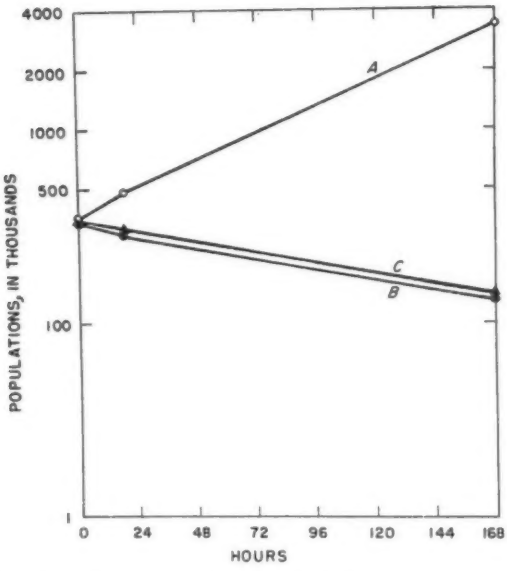
TEXT-FIGURE 1.—Time course of the cell populations for experiment 1. Initial populations 350,000.

7th, and 10th days. Used medium was cropped from the cultures for chemical analyses at 17-hour, 7-day, and 14-day intervals when the populations in the cultures were also enumerated. Acellular medium was also incubated for these intervals. Chemical analyses were made on pooled, used fluid from a minimum of 4 flasks and on the acellular incubated medium.

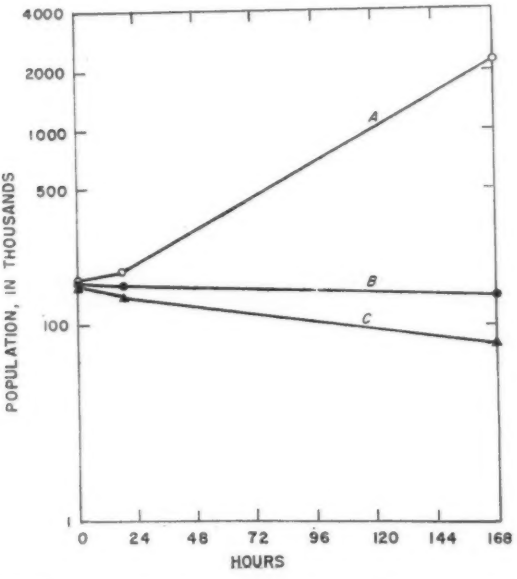
Glucose was estimated by an adaptation of the Sumner method (*17*). Lactic acid was estimated as previously described (*18*). α-Keto acids were examined by paper chromatography following solvent separation, as before, except that it was not necessary to use ultrafiltrates (*18*). For estimation of gross total α-keto acids in terms of pyruvic acid the method was modified as follows (*18*): One ml. of the medium was added to 50 ml. water and 2 ml. of 2 N HCl containing 2 mg. dinitrophenylhydrazine was then introduced. The well-mixed solution was allowed to stand at 25° C. for 30 minutes and the hydrazones were extracted as before (*18*). Sodium hydroxide was added to the extract to a final normality of 0.7; the concentration in the red hydrazone solution was ascertained at 440λ. Gross total amino acids were estimated with ninhydrin (*19*); glutamine and ammonia were estimated by the Conway-Butler procedure (*20*). The cells were grown in T-15 flasks in 3 ml. medium per flask. At each fluid change 2 ml. of the used fluid was withdrawn and replaced with 2 ml. of fresh medium.

Results

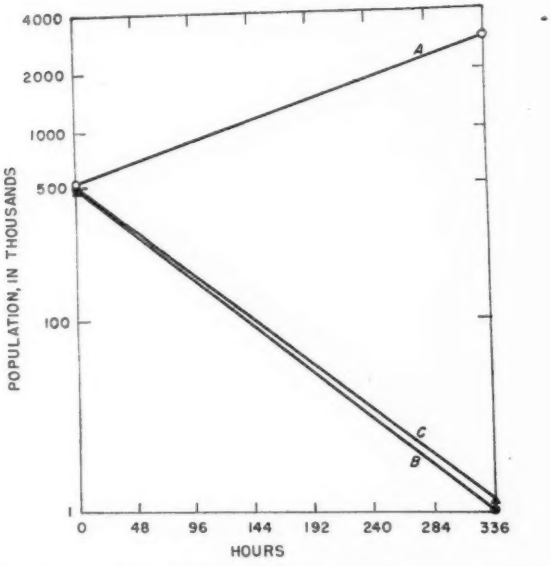
The average inoculum population per flask and the populations at 17 hours and at 7 days for experiments 1, 2, and 3 are shown in text-figures 1, 2, and 3. Text-figure 4 gives the initial and final populations for the cultures maintained for 14 days. It is apparent from all studies that in the cultures lacking only added glutamine (B) and glutamine and asparagine (C) populations fell below those of the inoculums and far below those of the controls at the 7- and 14-day intervals. With the complete medium (A) the population increased a minimum of 3 times over the inoculum. Text-figure 5 shows the gross total disappearance of glucose in mg. per 100 ml. from the medium and the excess lactic acid produced in the medium during the interval. Total α-keto acid concentration at the time of sampling is also shown. The keto acid values are calculated by reference to a pyruvic acid standard. Actually only about 60 percent of the amount is pyruvic, with α-ketoglutaric acid representing about 20 percent and the rest apparently being made up of traces of several acids such as α-ketoisocaproic and α-ketoisovaleric acid. Table 1 lists the gross total quantity of glucose used per culture and the mg. glucose used per million cells. It will be noted that, while the cultures without glutamine added used very little glucose, when the calculation is based on the few cells remaining alive in the deficient medium their utilization of glucose per cell compares with the growing cultures. Little of the α-keto acids accumulates in the used fluid in any of the experiments. Experiment 4 is not included in the results listed in text-figure 5 or table 1 because, at the time of final



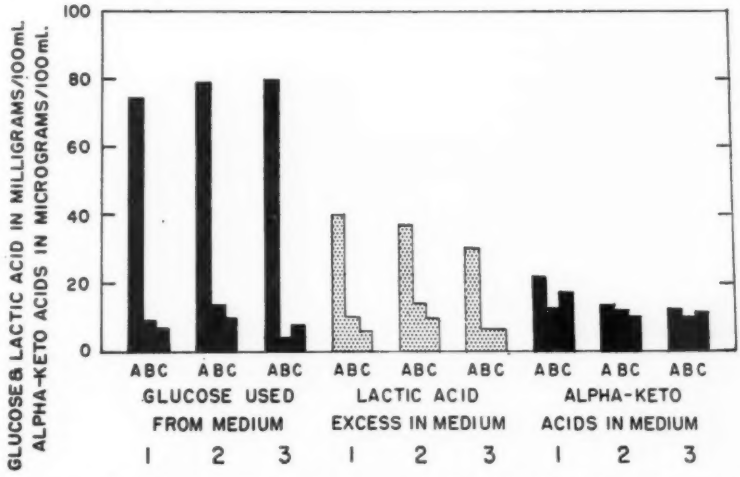
TEXT-FIGURE 2.—Time course of the cell populations for experiment 2. Initial populations 400,000.



TEXT-FIGURE 3.—Time course of the cell populations for experiment 3. Initial populations 160,000.



TEXT-FIGURE 4.—Time course of the cell populations for experiment 4. Initial populations 540,000.



TEXT-FIGURE 5.—Glucose utilization and excess lactic acid and α-keto acids appearing in the mediums for experiments 1, 2, and 3.

sampling, all the glucose had been used from the medium by the cells in the glutamine-containing fluid. It was considered invalid to calculate rates under such circumstances.

Results of the analyses for lactic acid are shown in table 2. There was no lactic acid in the medium supplied to the cells and very little was

TABLE 1.—*Utilization of glucose from protein-free medium NCTC 109 by cells of strain NCTC 2071 in the presence and absence of glutamine—last 4 experimental days*

Experiment and set	Total glucose		Populations* ($\times 10^{-6}$)	Utilization per million cells (*24 hours*)
	Supplied (*mg.*)	Used (*mg.*)		
2879 (1)				
A	2. 5	2. 08	1. 80	0. 29
B	2. 95	0. 08	0. 17	0. 12
C	2. 90	0. 18	0. 19	0. 24
2880 (2)				
A	2. 5	2. 08	1. 88	0. 28
B	2. 90	0. 35	0. 40	0. 22
C	2. 92	0. 21	0. 29	0. 18
2883 (3)				
A	2. 5	1. 99	1. 05	0. 47
B	2. 95	0. 05	0. 15	0. 08
C	2. 9	0. 18	0. 095	0. 47

*Population numbers have been adjusted to mid-period on the assumption that the growth curve is continuous and linear as shown in the graphs. Experiment 4 is omitted from this table, and also tables 2 and 3, because all the glucose supplied to the cells was used from the medium in the final period.

found in the B and C flasks. Again, as in the glucose data, when that produced in the flasks is expressed in quantity per million cells the resulting values are larger than when much greater actual quantities were produced. This finding is doubtless an artifact rising from the rapidly decreasing populations and the consequent uncertainty of the denominator of the fraction used in the calculation.

The behavior of glutamine under various conditions is shown in table 3. The data are for a typical experiment, specifically number 3. Under

TABLE 2.—*Total quantity of lactic acid in medium in experiments 1, 2, and 3 recorded as milligrams per culture and as milligrams per million cells; period covered is last 4 days of experimental periods in text-figures 1, 2, and 3*

Experiment and set	Total lactic acid (*mg.*)	Population of culture ($\times 10^{-6}$)	Lactic acid per million cells (*mg./24 hours*)
2879 (1)			
A	1. 03	1. 80	0. 14
B	0. 28	0. 17	0. 41
C	0. 22	0. 19	0. 29
2880 (2)			
A	1. 00	1. 88	0. 13
B	0. 40	0. 40	0. 25
C	0. 33	0. 29	0. 28
2883 (3)			
A	0. 87	1. 05	0. 21
B	0. 2	0. 15	0. 33
C	0. 2	0. 095	0. 55

"A fresh, 96 hours" it will be noted that there is very little breakdown of the glutamine in the medium in the absence of cells. This is in agreement with our previous finding (*11*) and with the recent report of Levintow *et al.* (*8*) who used a different medium. Both of the samples in "A" (at 17 and 96 hours) indicate loss of glutamine from the medium. By comparison with the acellular medium this loss has to be attributed to the metabolic activity of the cells. It will also be noted in the table that the B and C fresh mediums yield a very small amount of ammonia in the procedure. As before (*11*), this appears to be attributable to the other amines in the medium.

TABLE 3.—*Glutamine in cultures at various intervals for the different sets of experiment 3. Micrograms amide N per culture at stated intervals*

Medium	Incubation time (*hours*)	Total N as NH_3 plus amide N		Total N found as NH_3	Total N as glutamine
		Taken	Found		
A fresh	96*	45	47	7	40
A used	96	45	48	29	19
A used	17	45	48	10	38
B fresh	96	0	4	3	1
B used	96	0	13	4	9
B used	17	0	10	3	7
C used	96	0	4	3	1
C used	96	0	15	4	11
C used	17	0	17	4	13

*It is to be noted that the 96-hour interval of this table represents that time since fluid change and is the period ending at 168 hours of text-figure 3.

Discussion

The strain of mouse fibroblast used in this work, NCTC 2071, had been maintained for 30 months in a protein-free synthetic medium. Because it is the only strain in the laboratory so far adapted to continuous growth in defined medium with no possibility for getting amino acids or unknown growth factors from added serum, it offers unique opportunity for study. Since there had been some difficulty in getting the strain to establish itself under these circumstances (*15*), it was thought that it might have changed in its requirements for continuous proliferation compared to its parent strain 929. Evidence is accumulating that amino acid requirements, in particular, may vary from strain to strain. Thus Neuman and McCoy (*7*) found asparagine as well as glutamine required by Walker carcinosarcoma 256 cells *in vitro*. Haff and Swim (*9*, *10*) have found that their strain of RM 3–56 rabbit fibroblasts needs serine in addition to those amino acids frequently considered necessary to the growth of mammalian cells in the presence of protein added with the medium (*5*). Morgan and Morton (*6*) used fresh chick-heart muscle and subjected it to an intracellular depletion of glutamine by carrying the tissue for 3 days in Hanks' (*21*) balanced salt solution and could not demon-

strate a requirement for glutamine. The difference in culture conditions as well as the difference in cell source may have some bearing on the results. The difference they found in lack of requirement for glutamine by chick-heart muscle appears to be one of the few instances of species difference shown by cells *in vitro*. The work reported herein indicates that the strain 2071 maintains a high requirement for glutamine similar to the 1469 strain of mouse liver origin. It shows this need very early, in fact within 17 hours of the time of transfer to a glutamine-free medium despite the ability of cells to concentrate and hold within themselves amino acids against a gradient, as found by Christensen *et al.* (*22*, *23*) and by Wiseman and Ghadially (*24*). The small amounts of glutamine found in the used fluid from the B and C categories of table 3 must represent amine stored by the cells and returned to the medium by the destruction of the cells and to some extent by diffusion, as already mentioned. Diffusion [4] out from the cell in the absence of metabolic activity appears to be very slight as was indicated by an additional experiment. When cells were washed and placed in Earle's balanced saline without any of the other constituents of NCTC 109 present in 4 hours at 0° C., there was less than 1 microgram of glutamine, in the medium, yielded by the cells. It is probably the small quantity of glutamine available from death of the cells in the B and C categories and that from diffusion which makes it possible for the few surviving cells to use glucose. The relatively large quantity of ammonia found in the used medium of the 96–hour experiment recorded under A of table 3 indicates that the ammonia from the amide group of the glutamine may not be used as the cell incorporates the glutamine into its protein. This accumulation of ammonia may well be a source of toxicity in the medium and could be one factor that makes frequent fluid changes with the cultures necessary. A similar ammonia accumulation under like circumstances was noted some years ago (*11*) with cells of clone 1469 of liver origin and of the NCTC 1795 hepatoma strain derived therefrom, and has been found in a different medium with human cells of the HeLa strain by Levintow *et al.* (*8*). The liberation of ammonia could point to a glutaminase activity of the cell since there was no glutaminase activity detectable in the medium; in fact there was no detectable protein in the medium of A categories of table 3. There was protein in the centrifuged, cell-free, used medium of the B and C categories, presumably representing cellular debris. No information is available on glutaminase activity of the cells.

The earlier studies by Sanford *et al.* (*13*) indicated that when strain 929 was the cell source and glutamine was omitted from medium 109, the cells grew at a rate barely significantly lower than that of the control cells in the medium containing glutamine. From these short-term experiments glutamine did not appear to be essential for limited growth if there was a satisfactory supply of other amino acids. We are inclined to ascribe the difference in those results and the results of the present

[4] Diffusion from the cell of free amino acids or ninhydrin-positive substances during metabolic activity of the cells has been noted previously (*18*, *25*).

study to the difficulties of depleting the cells of needed ingredients rather than to definite differences in the nutritional needs of strain 929 and its substrain 2071.

The total keto acids of text-figure 5 are very low in amount. The quantity is decidedly less than was found in the similar experiment with mouse cells of liver origin already cited (*11*). The earlier finding that liver cells accumulate a greater amount of keto acids in the medium compared to the findings with 2071 cells could seem to indicate an interesting quantitative difference in the two strains. However, it is known from other work (*26*) that the culture conditions to some extent influence the concentrations of the α-keto acids found.

References

(*1*) Ehrensvärd, G., Fischer, A., and Stjernholm, R.: Protein metabolism of tissue cells in vitro. 7. The chemical nature of some obligate factors of tissue cell nutrition. Acta physiol. scandinav. 18: 218–230, 1949.

(*2*) Morgan, J. F., Morton, H. J., and Parker, R. C.: Nutrition of animal cells in tissue culture. Proc. Soc. Exper. Biol. & Med. 73: 1–8, 1950.

(*3*) White, P. R., and Waymouth, C.: Chemically defined synthetic nutrients for tissue cultures for pharmacological studies. Ann. New York Acad. Sc. 58: 1023–1028, 1954.

(*4*) Pasieka, A. E., Morton, H. J., and Morgan, J. F.: The metabolism of animal tissues cultivated *in vitro*. I. Amino acid metabolism of chick embryonic-heart fibroblasts cultivated in synthetic medium M150. J. Nat. Cancer Inst. 16: 995–1009, 1956.

(*5*) Eagle, H., Oyama, V. I., Levy, M., Horton, C. L., and Fleischman, R.: The growth response of mammalian cells in tissue culture to L-glutamine and L-glutamic acid. J. Biol. Chem. 218: 607–616, 1956.

(*6*) Morgan, J. F., and Morton, H. J.: The nutrition of animal tissues cultivated *in vitro*. IV. Amino acid requirements of chick embryonic heart fibroblasts. J. Biophys. & Biochem. Cytol. 5: 145–150, 1957.

(*7*) Neuman, R. E., and McCoy, T. A.: The dual requirement of Walker carcinosarcoma 256 in vitro for asparagine and glutamine. Science 124: 124–125, 1956.

(*8*) Levintow, L., Eagle, H., and Piez, K. A.: The role of glutamine in protein biosynthesis in tissue culture. J. Biol. Chem. 227: 929–941, 1957.

(*9*) Haff, R. F., and Swim, H. E.: The amino acid requirements of rabbit fibroblasts, strain RM3–56. J. Gen. Physiol. 41: 91–99, 1957.

(*10*) Swim, H. E., and Parker, R. F.: The amino acid requirements of human uterine fibroblasts, strain U 12. (Abstract.) Anat. Rec. 124: 499, 1956.

(*11*) Westfall, B. B., Evans, V. J., Peppers, E. V., Hawkins, N. M., and Earle, W. R.: Effect of glutamine on the growth and metabolism of liver cells *in vitro*. J. Nat. Cancer Inst. 17: 131–138, 1956.

(*12*) Evans, V. J., Fioramonti, M. C., Sanford, K. K., Earle, W. R., and Westfall, B. B.: The effect of amino acids on the proliferation of long-term strain 2071 of NCTC 929. Am. J. Hyg. In press.

(*13*) Sanford, K. K., McQuilkin, W. T., Fioramonti, M. C., Evans, V. J., and Earle, W. R.: Study of amino acid requirements for increase in cell population of NCTC clone 929 (strain L). J. Nat. Cancer Inst. 20: 775–785, 1958,

(*14*) Evans, V. J., Earle, W. R., Sanford, K. K., Shannon, J. E., and Waltz. H. K.: The preparation and handling of replicate tissue cultures for quantitative studies. J. Nat. Cancer Inst. 11: 907–927, 1951.

(*15*) McQUILKIN, W. T., EVANS, V. J., and EARLE, W. R.: The adaptation of additional lines of NCTC clone 929 (strain L) cells to chemically defined protein-free medium NCTC 109. J. Nat. Cancer Inst. 19: 885–907, 1957.

(*16*) SANFORD, K. K., EARLE, W. R., EVANS, V. J., WALTZ, H. K., and SHANNON, J. E., JR.: The measurement of proliferation in tissue cultures by enumeration of cell nuclei. J. Nat. Cancer Inst. 11: 773–795, 1951.

(*17*) HENDRIX, J. P., WESTFALL, B. B., and RICHARDS, A. N.: Quantitative studies of the composition of glomerular urine. XIV. The glomerular excretion of inulin in frogs and necturi. J. Biol. Chem. 116: 735–747, 1936.

(*18*) WESTFALL, B. B., PEPPERS, E. V., and EARLE, W. R.: The change in concentration of certain constituents of the medium during growth of the strain HeLa cells. Am. J. Hyg. 61: 326–333, 1955.

(*19*) MOORE, S., and STEIN, W. H.: Photometric ninhydrin method for use in the chromatography of amino acids. J. Biol. Chem. 176: 367–388, 1948.

(*20*) BUTLER, G. W.: Determination of glutamine and asparagine in plant tissue extracts. Anal. Chem. 23: 1300–1304, 1951.

(*21*) HANKS, J. H., and WALLACE, R. E.: Relation of oxygen and temperature in preservation of tissues by refrigeration. Proc. Soc. Exper. Biol. & Med. 71: 196–200, 1949.

(*22*) CHRISTENSEN, H. N., and RIGGS, T. R.: Concentrative uptake of amino acids by the Ehrlich mouse ascites carcinoma cell. J. Biol. Chem. 194: 57–68, 1952.

(*23*) CHRISTENSEN, H. N., RIGGS, T. R., Fischer, H., and PALATINE, I. M.: Amino acid concentration by a free cell neoplasm: Relations among amino acids. J. Biol. Chem. 198: 1–15, 1952.

(*24*) WISEMAN, G., and GHADIALLY, F. N.: Studies in the amino acid uptake of RD3 sarcoma cell suspensions *in vitro*. Brit. J. Cancer 9: 480–485, 1955.

(*25*) MORTON, H. J., PASIEKA, A. E., and MORGAN, J. F.: The nutrition of animal cells cultivated in vitro. III. Use of a depletion technique for determining specific nutrition requirements. J. Biophys. & Biochem. Cytol. 2: 589–596, 1956.

(*26*) WESTFALL, B. B., EVANS, V. J., PEPPERS, E. V., HAWKINS, N. M., BRYANT, J. C., SCHILLING, E. L., and EARLE, W. R.: Observations on the metabolic behavior of a clone of mouse liver cells grown in agitated fluid suspension. Cancer Res. 16: 1958.

ANNOUNCEMENT

A Clinical Conference on Cancer Chemotherapy

The University of Texas M. D. Anderson Hospital and Tumor Institute and The University of Texas Postgraduate School of Medicine present a Clinical Conference on Cancer Chemotherapy, November 14 and 15, 1958.

The third annual clinical conference for Texas Physicians will be held Friday, November 14, and Saturday, November 15, 1958, at The University of Texas M. D. Anderson Hospital and Tumor Institute, Texas Medical Center, Houston.

Registration: 8:30–9:00 a. m., Friday, November 14, at the auditorium of M. D. Anderson Hospital. Hotel reservations should be made well in advance of the Clinical Conference.

Hours: Members of the Academy of General Practice desiring credit for attendance at this conference must register in advance with the University of Texas Postgraduate School of Medicine, Jesse Jones Library Building, Houston, Texas. Eight hours will be certified for attendance at this conference. A $5.00 registrar's fee must accompany the registration for the Academy. Make checks payable to The University of Texas.

U. S. GOVERNMENT PRINTING OFFICE: 1958